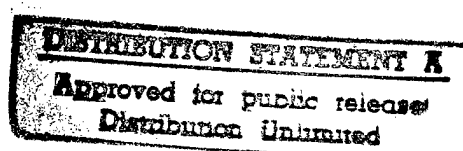


**NEURAL RESPONSES TO INJURY:
PREVENTION, PROTECTION, AND REPAIR
Annual Technical Report
1996**

Submitted by

Nicolas G. Bazan, M.D., Ph.D.
Project Director



Period Covered: 20 September, 1995, through 19 September, 1996

Cooperative Agreement DAMD17-93-V-3013

between

United States Army Medical Research and Development Command
(Walter Reed Army Institute of Research)

and

Louisiana State University Medical
Center
Neuroscience Center of Excellence

Volume 3 of 9

**The Neuro-
immunology of
Stress, Injury and
Infection**

Project Directors:
Bryan Gebhardt, Ph.D.
Daniel J.J. Carr, Ph.D.

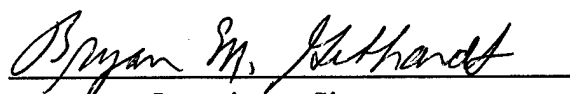
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ANIMAL USE
SEPTEMBER 20, 1995 THROUGH JULY, 1996

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: The Neuroimmunology of Stress, Injury, and Infection**, are as follows:

Species	Number Allowed	Number Used	LSU IACUC#
Mouse	360	360	1019


Investigator Signature

Volume 3 The Neuroimmunology of Stress, Injury, and Infection

Project Directors: Bryan Gebhardt, Ph.D.

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Halford WP, Gebhardt BM, Carr DJJ (1995) Functional role and sequence analysis of a lymphocyte orphan opioid receptor. *J Neuroimmunol* 59:91-101.

Halford WP, Gebhardt BM, Carr DJJ (1996) Mechanisms of Herpes Simplex Virus Type 1 Reactivation. *J Virology* 70:5051-5060.

Halford WP, Gebhardt BM, Carr DJJ: Persistent Cytokine Expression in Trigeminal Ganglion Latently Infected with Herpes Simplex Virus Type 1. *J Immunology*. In press.

Halford WP, Gebhardt BM, Carr DJJ (1995) Analysis of the immune response during stress-induced reactivation of herpes simplex virus type 1 from latency. The 9th International Congress of Immunology, July 23-29, San Francisco, CA, pp. 128.

Halford WP, Serou M, Gebhardt BM, Carr DJJ (1995) Functional Role and Sequence Analysis of a Lymphocyte Orphan Opioid Receptor. The 9th International Congress of Immunology, July 23-29, San Francisco, CA.

Project Director: Daniel Carr, Ph.D.

COOPERATIVE AGREEMENT NO. DAMD17-93-V-3013, Title: Neural
Response to Injury, Prevention, Protection, and Repair

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Carr DJJ, Serou M (1995) Exogenous and endogenous opioids as biological response modifiers.
Immunopharmacol 31:59-71.

Halford WP, Gebhardt BM, Carr DJJ (1996) Mechanisms of herpes simplex virus type 1 reactivation. *J Virol* 70:5051-5060.

Carr DJJ, Rogers TJ, Weber RJ (1996) The relevance of opioids and opioid receptors on immunocompetence and immune homeostasis. *Proc Soc Exp Biol Med*. In press.

Halford WP, Gebhardt BM, Carr DJJ (1996) Persistent cytokine expression in trigeminal ganglion latently infected with herpes simplex virus type 1. *J Immunol*. In press.

Carr DJJ (1996) Corticosterone is not associated with acute morphine-mediated suppression of peritoneal cytotoxic T lymphocyte activity in alloimmunized mice. *Drug Alcohol Depend*. Submitted.

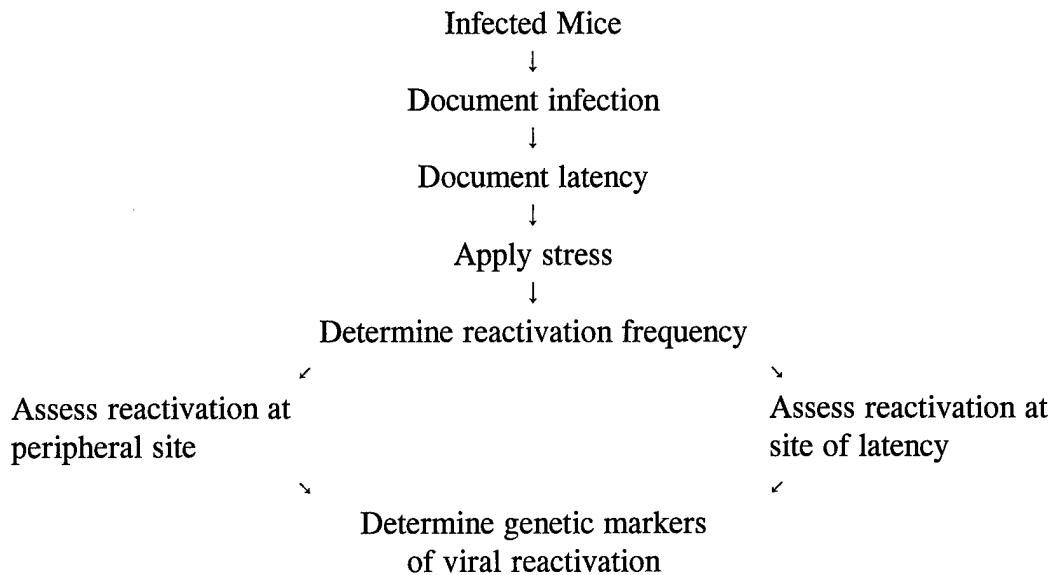
Halford WP, Scott M, Carr DJJ (1996) Cytokine expression in HSV-1 latently infected trigeminal ganglion cell cultures. *Virol*. Submitted.

Carr DJJ, Halford WP (1996) Lymphocytes delay kinetics of HSV-1 reactivation from *in vitro* explants of latently infected trigeminal ganglia (TG). 3rd International Congress of the International Society for Neuroimmunomodulation, Nov. 13-15, Bethesda, MD.

ABSTRACT

The hypothesis on which this investigation is based is that stressors such as transient temperature changes and restraint signal the central nervous system eliciting the release of catecholamines and adrenal steroids which, in turn, affect the immune system resulting in the reactivation of latent viruses. Employing a mouse model of stress-induced reactivation of herpes simplex virus type 1 (HSV-1), we are determining the time course of viral reactivation relative to the alteration of immune parameters including lymphocyte functions and numbers. Specifically, we are correlating the expression of various immunomodulatory cytokine genes with the levels of neuroendocrine monoamines, as well as the activation of the hypothalamic-pituitary-adrenal (HPA) axis and relating these to the reactivation of infectious virus in the nervous system. Alterations in serum corticosterone and shifts in monoamines in the brains, trigeminal ganglia, and brain stems of latently infected and reactivated mice following the application of stress are being studied. Differences between control (not stressed) and stressed animals are being determined relative to the incidence of viral reactivation and the affect of stress on immunological regulation of the reactivation process. The knowledge gained from this investigation will provide an understanding of the interaction between the nervous system, the neuroendocrine system, and the immune system during times of stress at the molecular and cellular levels.

FIRST YEAR FLOW CHART



First Year Goal: To determine the effects of a brief period of thermal stress (10 minutes at 43°C) and restraint stress (60 minutes) as indirect mediators of HSV-1 reactivation from neural tissues.

These experiments were designed to allow us to determine the frequency of viral reactivation following moderately stressful events and to establish a baseline from which we can launch a full-scale assault on the analysis of the neuroendocrine-immunologic interactions which take place during stress-induced viral reactivation. Groups of 10 mice in each group, which had been infected with the McKrae strain of HSV-1 by the ocular route 35 days previously were subjected to one of the stress protocols. At 24 hours after the application of the stressor (43°C for 10 minutes or 60 minutes of restraint stress) the groups of mice were sacrificed, the ocular surface swabbed, the globes removed, and the trigeminal ganglia dissected free. Control groups of animals included uninfected, stressed animals, infected animals that were not stressed, and animals which were neither infected nor stressed.

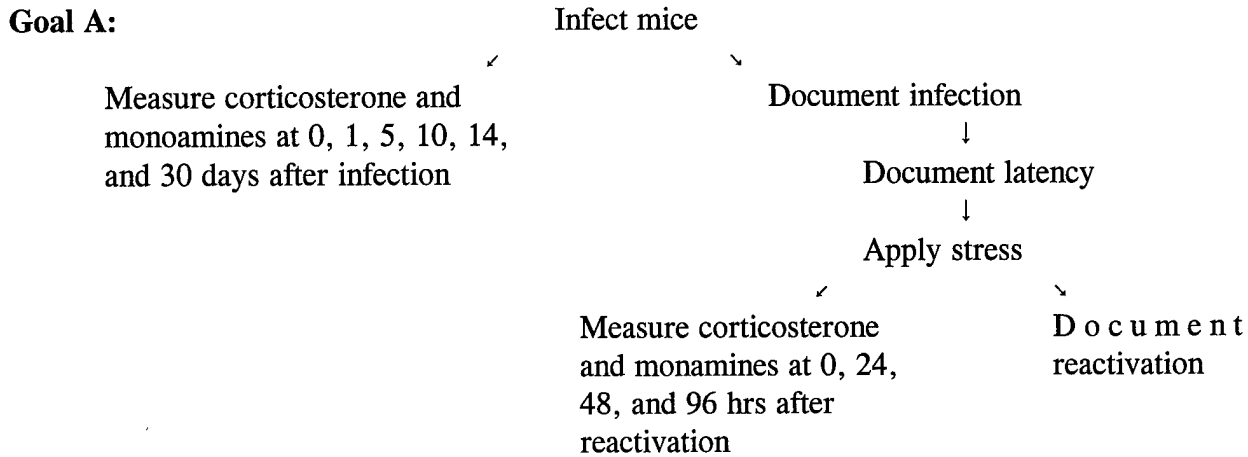
Table 1 below indicates the results of the assays for infectious virus from the experimental and control groups of animals. For the convenience of the presentation of the data, the results obtained with animals subjected to the two different stress paradigms are separated into Tables 1 and 2. It can be seen that the heat stress paradigm induced a higher percentage of reactivations (80%) as compared to the restraint stress model (40%). This experiment was repeated three times in order to confirm the results and to permit detailed statistical analyses. By analysis of variance (ANOVA) the difference between the reactivation of infectious virus in the tears, ocular tissue, and trigeminal ganglia of infected, stressed animals was significantly different from any of the control groups ($P < 0.005$). Thus, the stressors chosen for use in these investigations are bonafide methods of inducing viral reactivation.

TABLE 1: Viral Reactivation Following Heat Stress			
Treatment Groups	Presence of Infectious Virus In:		
	Tear Film	Corneas	Trigeminal Ganglia
Infected, stressed	4/10	6/10	8/10
Infected, not stressed	0/10	0/10	0/10
Not infected, stressed	0/10	0/10	0/10
Not infected, not stressed	0/10	0/10	0/10

TABLE 2: Viral Reactivation Following Restraint Stress			
Treatment Groups	Presence of Infectious Virus In:		
	Tear Film	Corneas	Trigeminal Ganglia
Infected, stressed	1/10	3/10	4/10
Infected, not stressed	0/10	0/10	0/10
Not infected, stressed	0/10	0/10	0/10
Not infected, not stressed	0/10	0/10	0/10

As a direct consequence of these studies comparing the efficiency of heat and restraint stress on viral reactivation, several manuscripts have been prepared. Most importantly it has been documented that herpes virus reactivation can be accomplished with a frequency which is adequate for investigation in this overall project. We have conducted a series of studies testing various antiviral drugs and, in a particularly revealing investigation, have shown that β -adrenergic receptor blocker, propranolol, can suppress viral reactivation following heat stress treatment of latent mice. These investigations are being continued to further define the physiologic and molecular mechanisms of viral reactivation.

SECOND YEAR FLOW CHART



Second Year Goal: A. To determine the neuroendocrine mechanism of stress-induced viral reactivation by measuring corticosterone and monoamine levels in the serum and nervous tissues of latently infected, stressed animals.

The overall goal of the experiments conducted as part of this specific aim are to determine the types of neuromediators which are involved in viral reactivation and also which are involved in modulating the immune response so as to permit viral reactivation. To determine the possible interactions between latent HSV-1 infection and the stress response of mice, the induction of endocrine and paracrine mediators was compared in four treatment groups: infected, stressed mice; infected, not stressed mice; uninfected, stressed mice; and uninfected, not stressed mice. Using the heat stress paradigm described above, it was found that 24 hours after application of the stressor that serum corticosterone in the infected mice were significantly higher as compared to uninfected mice ($P < 0.05$). The data from a typical experiment in this series is shown in Table 3 below.

TABLE 3: Effect of Heat Stress-Induced Viral Reactivation on Corticosterone Levels*			
	HSV-1 Infected	Not Infected	Averages
Stressed	102.3	38.5	70.4
Not Stressed	63.1	52.2	57.7
Averages	82.7	45.3	
Two-way ANOVA: H_0 : Probability: $X_{av}(\text{Stressed}) = X_{av}(\text{Not Stressed})$ $p = 0.28$ $X_{av}(\text{Infected}) = X_{av}(\text{Not Infected})$ $p = 0.015$ *nanograms/ml			

It can be seen that there was a statistically significant elevation in corticosterone levels in the infected mice. Additional studies of the monoamine levels in the brain stems of the experimental and control groups of animals have been conducted 24 hrs after application of the stressor. Catecholamine levels were not found to be significantly altered in any of the treatment and control groups investigated to date (Table 4).

TABLE 4: Effect of Heat Stress-Induced Viral Reactivation on Brainstem Catecholamine Levels*				
Treatment Groups	NE‡	SHIAA	DA	5HT
Infected, stressed	931	228	31.3	220
Infected, not stressed	1030	244	88.3	280
Not infected, stressed	742	131	23.2	186
Not infected, not stressed	966	227	56.6	251
One-way ANOVA: $X_a = X_0 = X_1 = X_2$	$p = 0.77$	$p = 0.58$	$p = 0.17$	$p = 0.69$
‡NE = norepinephrine; SHIAA = 5-hydroxyindoacetic; DA = dopamine; 5HT = 5-hydroxytryptophane *picograms/milligram				

Further studies of the catecholamine and corticosterone levels at earlier time points and the

significance of this response to viral reactivation are in progress.

Our initial investigation into the modulation of the immune response mediated by neuroendocrine mediators as a response to heat stress has produced some meaningful results. Among other things, we have investigated the production of interleukin 6 (IL-6) at the cellular and molecular level following application of stress to latently infected mice. We have noted that serum concentrations of corticosterone and IL-6 in uninfected, stressed mice were positively correlated ($r = 0.87$), but were negatively correlated ($r = -0.28$) in infected, stressed mice. These data are shown in Table 5 below. This observation suggests that an underlying difference exists between the regulation of IL-6 and corticosterone when one compares latently infected and uninfected animals following exposure to the stressor.

TABLE 5: Correlation Coefficients of Serum Corticosterone and IL-6 Levels in Heat Stressed, Infected Animals	
Treatment Groups	r Values
Infected, stressed	$r = -0.28$
Infected, not stressed	$r = -0.57$
Not infected, stressed	$r = 0.87$
Not infected, not stressed	$r = 0.27$
T-test: $H_0: r_{(\text{infected/stressed})} = r_{(\text{not infected/stressed})}$	

Molecular biological analysis of the expression of the IL-6 gene in the trigeminal ganglion of stressed latently infected mice compared to stressed, uninfected mice, as well as mice that were not stressed but latently infected, reveal that there is an apparent reduction in the expression of the IL-6 message in the latently infected, stressed animals (Fig. 1).

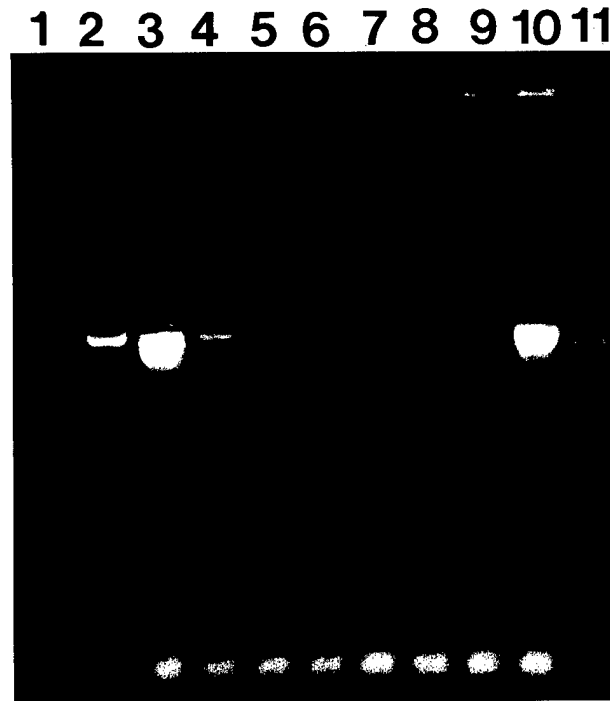
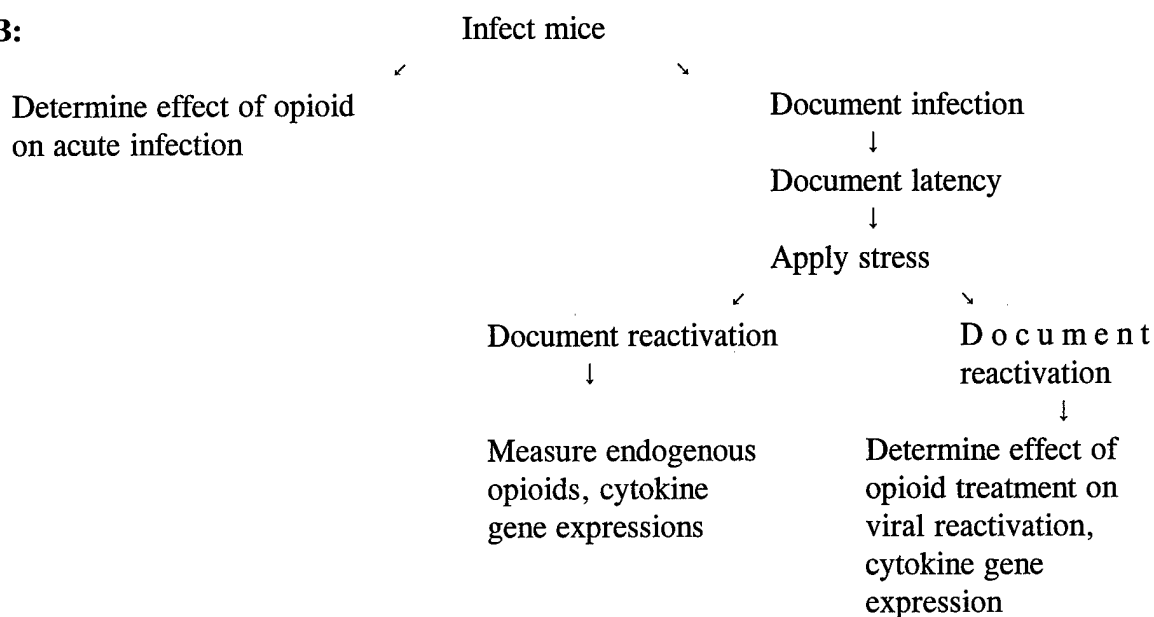


Figure 1. Reverse transcription-polymerase chain reaction (RT-PCR) for IL-6 mRNA in trigeminal ganglia. Lane 1 = Molecular weight markers; Lane 2 = Not infected, not stressed; Lane 3 = Not infected, stressed; Lanes 4,5 = Infected, not stressed; Lanes 6-9 = Infected, stressed; Lane 10 = Positive control; Lane 11 = Molecular weight markers.

Since the trigeminal ganglion is one of the sites of latency of HSV-1, we speculate that perhaps the virus can in some way alter the host's production of immunomodulatory mediators including the pro-inflammatory cytokine IL-6 and, by extension, corticosterone. Currently, competitive PCR is being carried out to quantitate differences between the levels of IL-6 expression in the trigeminal ganglion of the various treatment groups. We can now detect as few as 100 copies of template. Further studies of the role of cytokines (TNF- α , IL-1, and IL-6) and neuroendocrine mediators in viral reactivation during the host immune response are in progress.

SECOND YEAR FLOW CHART

Goal B:



Our investigations of the role of cytokine gene activation following heat stress have yielded a number of interesting results. A manuscript is in preparation detailing the time course of cytokine gene expression in the cornea and trigeminal ganglion of mice during acute, latent, and reactivated viral infection in mice. In essence, we have found that several of the pro-inflammatory cytokines are expressed in the cornea and trigeminal ganglion during acute viral infection and that subsequently interleukin 2, interleukin 10, and gamma interferon are produced once the infected host's immune system mobilizes lymphocytes which migrate into the sites of infection. Upon viral reactivation, we find that there is only a very transient expression of interleukin 6 and the chemokine RANTES, followed by a spike of gamma interferon production. Only if the viral reactivation is prolonged by manipulation of the host's immune response do we find anything more than a very brief burst of cytokine gene activity in the trigeminal ganglion following reactivation.

Second Year Goal: B. To investigate the role of the neuroendocrine system following morphine exposure.

The approach that we have taken to investigate the role of the neuroendocrine system in viral latency and reactivation has been through the use of hypothysectomized and adrenalectomized animals. In a study which is now being prepared for publication, we have found that adrenalectomy significantly impairs the development of antiviral immunity, both cellular and humoral, and as a result infectious virus is present in at both the ocular surface and in the trigeminal ganglion for longer periods of time. Hypothysectomized animals appear to respond relatively normally with the development of an antiviral immune response and further studies on the compensatory mechanisms involved are in progress. Studies of viral reactivation following application of the stress protocol are being pursued in such animals.

As a direct result of the Department of Defense support for our study, we have been able to provide new insights into the role of morphine-mediated suppression of cellular immunity in mice. These studies have culminated in the submission of four abstracts, the presentation or planned presentation of our findings at three national meetings, and the submission of four manuscripts. In addition, pertinent studies are near completion in the cloning of an orphan opioid receptor from lymphocytes which may lead to insights as to the role of opioids (exogenous and endogenous) on the regulation of immunocompetence either constitutively or following bacterial, parasitic, or viral infection. A summary of the findings to date are presented below.

Opioids, such as morphine, are a known chemical stressor that has a detrimental effect on the immune system. In fact, early research by Bryant, Bernton, and Holaday (1987; 1991) of the Division of Neuropsychiatry of Walter Reed Army Institute showed that morphine was a potent immunosuppressive drug which when administered *in vivo* suppressed mitogen-induced lymphocyte proliferation and delayed-type hypersensitivity reactions through the activation of the HPA axis. To further these initial observations, the P.I. (Gebhardt) and co-P.I. (Carr) began a

series of studies to investigate the potential role of the sympathetic nervous system and HPA axis involvement in morphine-induced suppression of cell-mediated immunity concentration on natural killer (NK) activity and the generation of cytotoxic T lymphocytes (CTLs). Both of these effector populations are involved in viral and tumor surveillance making them key figures in reducing the potential incapacitation as a result of viral reactivation or infection. The results of these studies showed that morphine suppressed NK activity through central activation of α -adrenoreceptors (Carr et. al, 1994). Additional studies were conducted on chronic morphine exposure and CTL activation using an allogeneic mouse model. The results of these studies show that chronic morphine exposure significantly suppresses CTL activity following alloimmunization in mice. The suppression is, in part, due to a reduction in intracellular signaling following effector - target cell conjugation as well as the release and synthesis of enzymes associated with the "lethal hit". Using a pharmacological approach, the suppression can be antagonized using the μ -opioid receptor antagonist, β -funaltrexamine, but not the δ -opioid receptor antagonist, BNTX. Likewise, serum measurements of corticosterone and DHEA from the vehicle- and morphine-treated mice suggested aberrant adrenal function in the morphine-treated animals. The biological significance of these results is substantiated by the study showing that mice infected with HSV-1 and exposed to morphine succumb sooner and with increased frequency, as compared to vehicle-treated, infected mice. These results have helped establish potential mechanisms at a systemic, cellular, and molecular level that may be altered following chronic morphine exposure.

The observations showing that morphine-induced suppression of immunocompetence is mediated through central (brain) pathways has led us to investigate the relevance of the hypothesized presence of the opioid receptor(s) on cells of the immune system. Recently, the P.I. and co-P.I. generated oligonucleotide primers specific for transmembrane 3 and 5 and the δ -opioid

receptor and used these oligonucleotides to detect potential transcripts in stimulated and unstimulated lymphocytes. By RT-PCR, a product of 381 pb was generated. Subsequent primers were made and used to obtain a full-length cDNA which has >90% sequence homology to an orphan opioid receptor cloned from mouse brain. Northern gel analysis of RNA obtained from splenic lymphocytes indicates the transcripts are present but in reduced numbers relative to brain extracts (Halford, Gebhardt, & Carr, manuscript in preparation). These results will be instrumental in establishing a direct link between the endogenous lymphocyte/macrophage-derived proopiomelanocortin hormones, the endorphins, and the potential autocrine- or paracrine-oriented feedback on cells of the immune system as well as the potential direct interaction between exogenous opioids (e.g., morphine) and cells of the immune system.

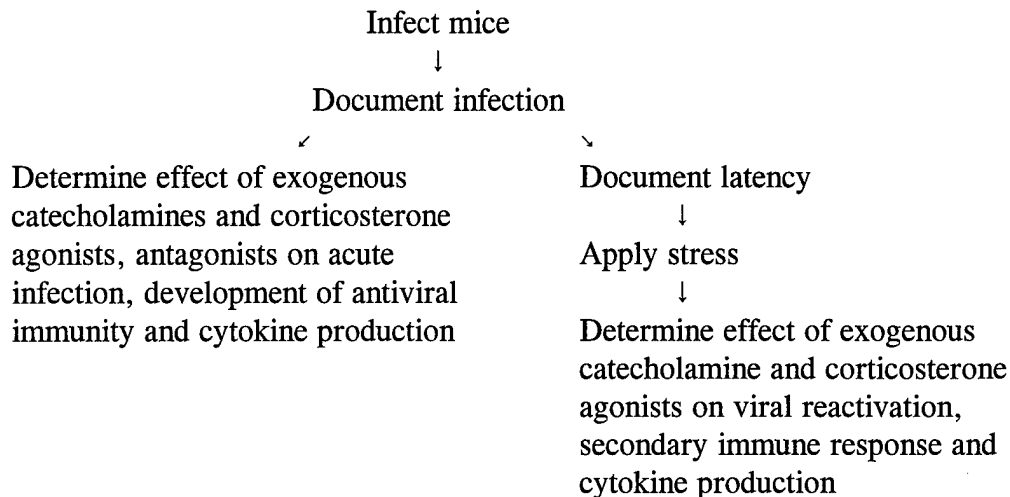
Currently, a kinetic study of the effects of morphine on CTL activity in alloimmunized mice is underway. In addition, the involvement of corticosterone in this model system is under analysis. The results would suggest that the acute administration of morphine to mice prior to alloimmunization dramatically suppresses peritoneal CTL activity. Serum corticosterone levels are elevated in the morphine administered animals relative to vehicle-treated controls at the 2 h time point but not 12 h or 120 h following morphine administration. To confirm the role of corticosterone involvement in the morphine-mediated suppression, the corticosterone synthesis inhibitor, cyanoketone is currently under study. The results of this investigation will be directly applicable to the studies involving viral reactivation and the neuroendocrine systems involved. Further studies regarding the role of peripheral and central α -adrenergic pathways in the reactivation of HSV-1 from latency and the effect on the immune system are in progress.

Third Year Goal: To determine the effect of externally applied catecholamines and

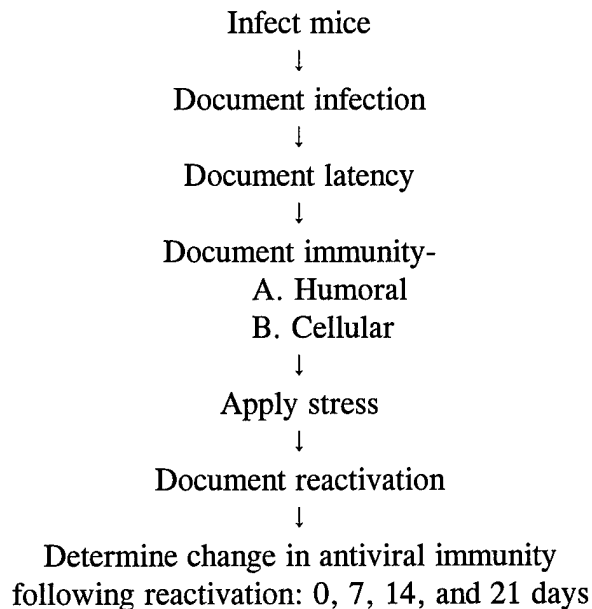
corticosterone antagonists and agonists in terms of their capacity to prevent or elicit viral reactivation in stressed and nonstressed animals.

THIRD YEAR FLOW CHART

Goal A:



Goal B:



We have made excellent progress in the first two years of this investigation. So much so that, in fact, investigations on the third year goals are beginning at the present time. We are

presently testing the levels of cellular and humoral immunity during latency and viral reactivation in pharmacologically normal mice. Shortly, investigations of the affects of exogenous catecholamines and corticosterone agonists and antagonists will begin. We fully expect that these studies will provide considerable insight into the role of catecholamines and corticosterone in the maintenance of latency and in viral reactivation following stress. Since we have already shown that the β -adrenergic receptor blocker, propranolol, can interfere with heat stress induced viral reactivation, we feel now that we are in an excellent position to determine the physiological and molecular linkages between stress and reactivation of herpes virus. The results of these studies should provide meaningful information regarding the development of new therapeutic paradigms for preventing viral reactivation in human beings.

Extensive studies have been conducted during the past grant year regarding the effect of exogenous catecholamine on acute infection, the development of antiviral immunity, and cytokine production in response to acute infection in mice. In addition, the effect of exogenous catecholamine on viral reactivation and the secondary immune response including cytokine production in mice has been studied. Dr. Daniel J.J. Carr, a co-investigator, has studied the effects of corticosterone and agonists and antagonists in a similar model of viral infection and reactivation.

Third Year Goal: A. Initially a series of experiments were conducted to determine the optimal does of the catecholamine, norepinephrine, on the neurophysiologic response of mice. It was important to determine a does of norepinephrine which would have an effect on the mouse's neuroendocrine system while not exerting a generalized toxic effect. Thus, in the first series of experiments groups of 5 mice per group were injected with norepinephrine in amounts ranging from 1 ng, 10 ng, 100 ng, 1 μ g, 10 μ g, 100 μ g, and 1 mg. At the time of the injection, the mice

were infected by the topical ocular route with herpes simplex virus type 1 (HSV-1). At days 3, 7, 12, 21, and 30 after infection the mice were bled and their serum antibody titers for HSV-1 determined. In addition, at days 3, 7, and 12 after infection some animals in each group were sacrificed for determining viral titers in their eyes and trigeminal ganglia.

Initial results indicated that the 1 mg and 100 μ g quantities of norepinephrine were toxic to mice (Table 6). It became evident that this concentration of norepinephrine injected intraperitoneally had a rapid debilitating effect on the mice and in the 1 mg concentration group, 5/5 animals died whereas 3/5 animals died in the 100 μ g injected group (Table 6).

Table 6: Dose-Response to Norepinephrine: Toxicity	
Treatment with Norepinephrine	Morbidity/Mortality/Total Animals
1 ng	0/0/5
10 ng	0/0/5
100 ng	0/0/5
1 μ g	0/0/5
10 μ g	0/0/5
100 μ g	0/3/2
1 mg	0/5/5

The surviving mice in each treatment group, as well as control, untreated mice which had been infected with herpes virus were tested for serum antibodies by using the enzyme linked immunosorbent assay (ELISA). It was noted that animals in the group treated with 10 μ g of norepinephrine had a lower average serum antibody titer to HSV-1 as compared to animals treated with smaller amounts of the drug or not treated at all (Table 7).

Table 7: Dose-Response to Norepinephrine: Serum Anti-HSV-1 Titers	
Treatment with Norepinephrine	Mean Optical Density at 405nm‡
1 ng	1.53
10 ng	1.49
100 ng	1.57
1 µg	1.44
10 µg	0.87*
100 µg	ND ^Δ
1 mg	ND
‡ELISA values using HSV-1 antigens. *This value is statistically significantly lower than the other values. ^Δ ND = Not determined.	

Investigation of the effect of the catecholamine, norepinephrine, on the cytokine response during acute viral infection was studied. Groups of mice were infected and simultaneously treated with 10 ng of norepinephrine intraperitoneally. This treatment was continued once daily for 7 days. Based on our previous investigations we have found that there is a significant increase in production of interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor α (TNF α) during acute viral infection. Groups of mice which had been infected and either not treated or treated with norepinephrine were sacrificed at days 7, 14, and 21 after infection. Serum concentrations of IL-1, IL-6, and TNF α and the presence of RNA transcripts for these cytokines in the eye and trigeminal ganglia were studied.

At 7 and 14 days after infection there was a statistically significant elevation in the concentration of IL-1, IL-6, and TNF α in the peripheral blood of infected, untreated mice. In comparison the norepinephrine treated mice had lower serum concentrations of these cytokines compared to control animals (Table 8).

Table 8: Effect of Norepinephrine on Serum Cytokine Titers‡										
Treatment		Interleukin-1*			Interleukin-6*			Tumor Necrosis Factor α *		
	Days After Infection	7	14	21	7	14	21	7	14	21
Norepinephrine		250	490	387	334	372	460	550	680	601
Placebo		180	1250	966	412	950	877	615	1480	1270
‡ELISA values in picograms. *Serum cytokine levels were statistically significantly lower in the norepinephrine treated animals at 14 and 21 days after infection.										

Analysis of ocular and peripheral nervous tissues for the presence of mRNA transcripts for IL-1, IL-6, and TNF α revealed that these cytokine transcripts were consistently present in infected animals compared to uninfected controls. Quantitative analysis of the level of transcript expression in norepinephrine treated, infected mice compared to infected, untreated mice is in progress.

Having determined that the catecholamine, norepinephrine, had an effect on the acute viral infection, and the initiation of an antiviral and cytokine response during viral infection, the next series of studies were designed to determine if the norepinephrine would have an impact on stress-induced viral reactivation and on the defensive response subsequent to viral reactivation. These studies have been carried out using groups of 5 mice in each that are injected with norepinephrine and a second group of saline-treated controls. Both groups of mice had been previously infected 40 days earlier with HSV-1 and the infection documented by performing ocular swabs and visual examinations of the ocular surface. Preliminary experiments suggested that the amount of virus, and therefore viral replication which occurred in the eye and trigeminal ganglia, was increased in norepinephrine mice compared to untreated mice. It was of interest and importance to study the

effect of this catecholamine on stress-induced viral reactivation. To this end, groups of latent mice were stressed, treated with norepinephrine, and the incidence of viral reactivation determined. In each experiment control animals latent for virus but not treated with norepinephrine were also studied.

Analysis of viral reactivation in control and norepinephrine-treated latent mice indicates that at the concentration tested the norepinephrine did not have a statistically significant effect on the incidence of viral reactivation following heat stress of the mice (Table 9). Further studies designed to analyze the humoral immune response and cytokine response following viral reactivation in control and norepinephrine-treated mice have revealed that there is no statistically significant difference between the two treatment groups. In all cases the antibody and cytokine (IL-1, IL-6, and TNF α) titers were statistically similar in the control and the norepinephrine-treated animals.

Table 9: Effect of Norepinephrine on Viral Reaction [‡]	
Treatment	No. Reactivations/Total*
Norepinephrine	8/12
	5/9
	6/14
Placebo	7/12
	5/10
	7/14
[‡] Latent mice were stressed (43°C water/10 min.) and the reactivation of virus determined 24 hours later. *Three replicate experiments; no statistically significant difference in number of animals exhibiting reactivation in the two treatment groups.	

Third Year Goal: B. Additional studies designed to determine if there is a change in the level of immunity following stress-induced viral reactivation have been conducted. Groups of mice latent for HSV-1 were subjected to heat stress and then the levels of cellular and humoral

immune responsiveness compared at 7, 14, and 21 days after viral reactivation. Control groups of mice include those that were latent for virus but not subjected to stress-induced reactivation. In terms of the serum antibody response to HSV-1, we find that there is no statistically significant change in the serum antibody titer to the virus following stress-induced viral reactivation (Table 10).

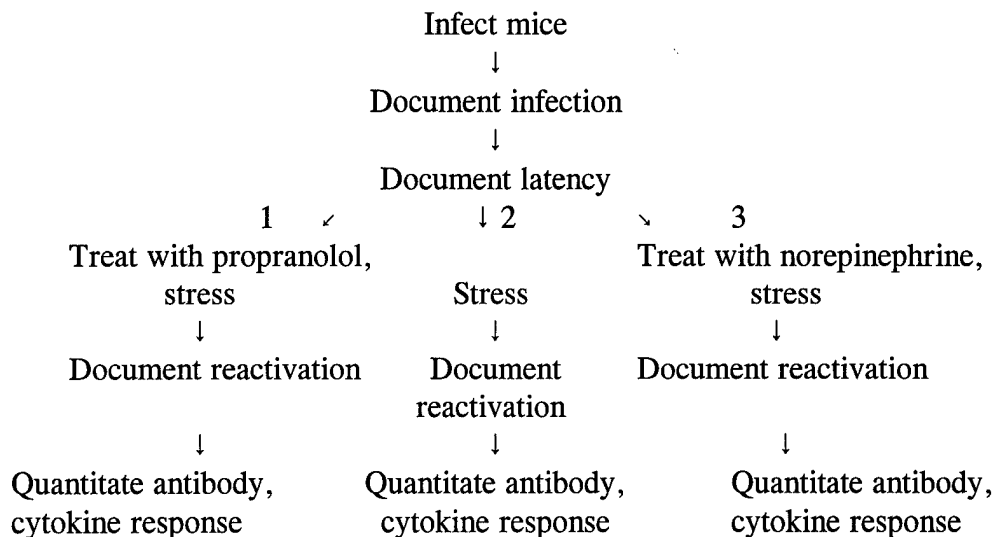
Table 10: Serum Anti-HSV-1 Titer Following Reactivation					
Treatment		Mean Optical Density at 405nm [‡]			
	Days After Reactivation	0	7	14	21
Stress-induced reactivation		1.41	1.26	1.14	1.29
No stress		1.22	1.30	1.16	1.25
[‡] ELISA values; there was no statistically significant difference between the serum antibody titers of the reactivated and control mice.					

Similarly, analysis of the cellular cytotoxic response of lymphocytes from virus-infected mice that have been induced to undergo reactivation fail to reveal a change in the frequency of cytotoxic T cell precursors in mice in whom viral reactivation has been induced as compared to control mice (Table 11).

Table 11: Cellular Cytotoxicity Following Reactivation [‡]					
Treatment		Peak Cellular Cytotoxicity (%)			
	Days After Reactivation	0	7	14	21
Stress-induced reactivation		14	21	20	24
No stress		15	18	24	26
[‡] Cell-mediated cytotoxicity of HSV-1, syngeneic target cells was performed using peripheral blood lymphocytes.					

Fourth Year Goal: To investigate the neuroimmunological relationship between stress-induced viral reactivation and recurrence of viral infection.

FOURTH YEAR FLOW CHART



We have made excellent progress in the first three years of this investigation. The studies proposed for the fourth year are shown diagrammatically in the Fourth Year Flow Chart (see above). These studies are designed to determine the pharmacologic mechanisms of stress-induced viral reactivation and, as well, to determine the interaction between the nervous system and the immune system in generating the ongoing antiviral response. We are particularly interested in determining if the catecholamines not only impact stress-induced viral reactivation, but in some way mediate, either directly or indirectly, an effect on the serum antibody and serum cytokine response. To this end, we will study control stressed mice, propranolol-treated stressed mice, and norepinephrine-treated stressed mice. We will investigate the frequency of reactivation in these groups, as well as quantitate serum antibody and serum cytokine responses in these groups of animals. From these data we hope to develop a comprehensive model of stress-induced viral

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reactivation from which we can propose therapeutic protocols which will effectively intervene in stress-induced viral reactivation in human beings.

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Halford WP, Gebhardt BM, Carr DJJ: Persistent cytokine expression in trigeminal ganglion latently infected with herpes simplex virus Type 1. In press: J Immunol, 1996.

PUBLICATIONS:

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2. Halford WP, Gebhardt BM, Carr DJJ: Mechanisms of Herpes Simplex Virus Type 1 Reactivation. *J of Virology* 70:5051-5060, 1996.
3. Halford WP, Gebhardt BM, Carr DJJ: Persistent Cytokine Expression in Trigeminal Ganglion Latently Infected with Herpes Simplex Virus Type 1. *J Immunology*. In press.

ABSTRACTS:

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Functional role and sequence analysis of a lymphocyte orphan opioid receptor [☆]

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Aims and Scope

The *Journal of Neuroimmunology* affords a forum for the publication of works applying immunologic methodology to the furtherance of the neurological sciences. Studies on all branches of the neurosciences, particularly fundamental and applied neurobiology, neurology, neuropathology, neurochemistry, neurovirology, neuroendocrinology, neuromuscular research, neuropharmacology and psychology, which involve either immunologic methodology (e.g. immunocytochemistry) or fundamental immunology (e.g. antibody and lymphocyte assays), are considered for publication. Works pertaining to multiple sclerosis, AIDS, amyotrophic lateral sclerosis, Guillain Barré syndrome, myasthenia gravis, and brain tumors form a major focus. The scope of the Journal is broad, covering both research and clinical problems of neuroscientific interest.

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(continued on inside back cover)

Functional role and sequence analysis of a lymphocyte orphan opioid receptor [☆]

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Abstract

Pharmacological evidence indicates that lymphocytes express opioid receptors, but this finding has been questioned. By DNA sequencing of reverse transcription-polymerase chain reaction products, we have found that mouse lymphocytes express mRNA encoding an orphan opioid receptor. These mRNA transcripts were detected in the CD4⁺, CD8⁺, and CD4[−]CD8[−] lymphocyte subpopulations. Northern blot analysis confirmed that splenic lymphocytes express a 1.5-kb orphan opioid receptor mRNA. Fifteen bases encoding Tyr⁷¹-Arg⁷⁵ in the first intracellular loop are alternatively spliced, suggesting that orphan opioid receptor mRNA encodes two receptor subtypes. Treatment of lipopolysaccharide-stimulated lymphocytes with orphan opioid receptor antisense oligonucleotides suppressed polyclonal IgG and IgM production by 50%. Our results provide direct evidence that lymphocytes express an opioid-like receptor gene, and suggest that this receptor plays a functional role in immunocompetence.

Keywords: Lymphocyte; Reverse transcription-polymerase chain reaction; Orphan opioid receptor; Antisense oligonucleotide; Alternative splicing

1. Introduction

Opioids have been shown to modify immune responses in vitro and in vivo. In vitro, opioid peptides suppress antibody production (Johnson et al., 1982; Heijnen et al., 1986; Taub et al., 1991), augment natural killer (NK) cell activity (Mathews et al., 1983), increase interferon (IFN)- γ production (Mandler et al., 1986), enhance monocyte-granulocyte chemotaxis (Van Epps et al., 1984), and promote CTL generation (Carr and Klimpel, 1986). These events are blocked by opioid receptor (OR) antagonists, implying that the immunoregulatory effects are mediated through OR–ligand interaction.

The characterization of ORs on cells of the immune

system has been problematic. Although early studies suggested the existence of opioid binding sites on leukocytes (Mehrishi and Mills, 1983; Falke et al., 1985), these studies were met with scepticism because unconventional experimental procedures were used and the data was not thoroughly analyzed (Sibinga and Goldstein, 1988). However, more recent studies have shown μ -like and κ opioid binding sites on lymphocytes and macrophages. These binding sites exhibit stereoselectivity, saturability, nanomolar affinity, and ligand-class preference (Madden et al., 1987; Carr et al., 1989; Ovadia et al., 1989; Bidlack et al., 1992). Photoaffinity-labeling techniques have identified δ - (Carr et al., 1988), κ - (Carr et al., 1989), and μ -like (Radulescu et al., 1991) opioid binding sites on leukocytes that structurally resemble their neuroendocrine counterparts (Carr, 1991). Moreover, in one study the purification of an opioid binding moiety that showed ligand selectivity was demonstrated (Carr et al., 1990a). However, the biological relevance of these binding sites remains in question.

[☆] This work does not necessarily reflect the position or the policy of the government, and no official endorsement should be inferred.

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The administration of opioids *in vivo* (e.g. morphine, the prototypic μ ligand) has been shown to alter NK cell activity by indirect routes including the activation of the periaqueductal gray matter of the mesencephalon (Weber and Pert, 1989) and α -adrenergic pathways (Carr et al., 1993; Carr et al., 1994). Other studies have shown morphine exposure results in thymic atrophy (Sei et al., 1991) and decreases in interleukin (IL)-2/IL-4 and IFN- γ production (Lysle et al., 1993). These effects are mediated by the hypothalamic-pituitary adrenal axis and peripheral β -adrenergic action. Taken together, the studies indicated the need to re-evaluate the existence and functional significance of ORs on cells of the immune system.

The development of molecular biological techniques has allowed investigators to clone a variety of neuropeptide receptors including brain δ -, κ -, and μ - ORs (Evans et al., 1992; Kieffer et al., 1992; Wang et al., 1993; Yasuda et al., 1993). Cloning of these ORs has led to the discovery of a new OR class, referred to herein as the orphan opioid receptor (oOR), which shares 66% nucleotide sequence homology with δ OR. oOR has been cloned from human (Mollereau et al., 1994), rat (Bunzow and Grandy, 1994; Chen et al., 1994; Fukuda et al., 1994), and mouse (Yasuda et al., 1994) brain cDNA libraries, but these studies have only begun to address the function of oOR.

Consistent with their role as G-protein coupled receptors, ORs possess seven transmembrane (TM) spanning regions whose amino acid sequences are highly conserved amongst δ -, κ -, and μ - ORs (Reisine and Bell, 1993). Based on this homology, oligonucleotide primers were prepared to the TM-encoding sequences of DOR-1, a δ OR clone (Evans et al., 1992), and reverse transcription polymerase chain reaction (RT-PCR) was used to screen splenic lymphocyte RNA for OR-encoding transcripts. However, these primers did not amplify δ OR mRNA, but rather, amplified the homologous portion of a mRNA that encodes oOR. Our results demonstrate that lymphocytes express oOR mRNA.

2. Materials and methods

2.1. Animals and cells

BALB/c, DBA/2, and ICR-Swiss mice (Harlan-Sprague Dawley, Indianapolis, IN) were used in these experiments. EL-4 mouse thymoma and L929 mouse fibroblast cell lines were obtained from the American Type Culture Collection (Rockville, MD). Lymphocyte suspensions were teased out of the spleen and drawn through a 23-gauge needle to mechanically disperse cells. RBCs were separated from lymphocytes either

with 0.84% NH_4Cl , or by gradient centrifugation over Ficoll-Hypaque (Lympholyte M[®], Accurate Scientific and Chemical Co., Westbury, NY). Concanavalin A (ConA, 10 $\mu\text{g}/\text{ml}$) stimulation of splenic lymphocytes was performed in RPMI 1640, 10% fetal bovine serum for 48 h at 37°C/5% CO_2 at a density of 5×10^6 cells per ml.

2.2. Isolation of RNA

RNA extraction was performed with Ultraspec[®] RNA isolation reagent (Biotecx Inc., Houston, TX) according to the manufacturer's instructions. RNA was isolated from mouse brain and whole spleen by homogenization of the entire organ in Ultraspec[®]. RBC lysis was performed prior to the isolation of poly(A)⁺ RNA from splenic lymphocytes. For RT-PCR and total RNA blots, lymphocytes were purified with Lympholyte M[®] prior to RNA extraction. Poly(A)⁺ RNA was purified from total RNA using a Poly ATtract[®] mRNA isolation kit (Promega Corp., Madison, WI).

2.3. Cell sorting by FACS

Spleen cells from ICR mice were collected by mechanical dispersion and RBCs were lysed with 0.84% NH_4Cl . The cells were washed with RDF buffer (R&D Systems, Minneapolis, MN) and labeled with rat anti-mouse CD4 (clone RM4-5, Pharmigen, San Diego, CA) conjugated with FITC and rat anti-mouse CD8 (clone 53-6.7, Pharmigen) conjugated with phycoerythrin. The cells were incubated on ice for 30 min and subsequently washed twice with RDF buffer. The labeled cells were then sorted using a Coulter Elite FACS (Coulter, Hialeah, FL). $\text{CD4}^+\text{CD8}^-$, $\text{CD4}^-\text{CD8}^+$, and $\text{CD4}^-\text{CD8}^-$ cells (5×10^6 cells) were collected for RT-PCR analysis. CD4^+ and CD8^+ populations were 98% pure as assessed by FACS.

2.4. RT-PCR and DNA sequencing

First strand cDNA was synthesized from 500 ng total RNA using an oligo-dT₁₅ primer and AMV reverse transcriptase (Promega Corp.) according to the manufacturer's instructions. The cDNA template was combined with buffer, 0.25 μM each PCR primer, 100 μM each dNTP, and 2.5 U *Taq* polymerase (Promega Corp.) in a 50- μl reaction volume and overlaid with mineral oil. PCR was accomplished in a MJ Research thermal cycler (Watertown, MA) with 35 cycles of 94°C (1'15") \rightarrow 57°C (1'15") \rightarrow 72°C (35"). Oligonucleotide PCR primers were synthesized by LSU Medical Center Core Laboratories (LSUMC, New Orleans, LA) and are listed in Table 1. PCR amplification with glyceraldehyde phosphate dehydrogenase primers (G3PDH, Iwai et al., 1991) was performed on an aliquot of each

Table 1
Delta and orphan opioid receptor PCR primers

PCR Primer	Oligonucleotide sequence
δ OR395 ⁺ (532)	5' GCT GTG CTC TCC ATT GAC TAC TAC AAC ATG
δ OR695 ⁻ (832)	5' CGA AGG CAA AGA GGA ACA CGC AGA T
oOR52 ⁺	5' TCA TTG TGC TCC TGC CTG CCT TTC T
oOR114 ⁺	5' GAG GTT GTG TGT GCT GTT GGA GGA A
oOR925 ⁻	5' GGT CCT TCT CTC GGG AGC CTG AAA G
oOR1292 ⁻	5' ATG GGC AGG TCC ACG CCT AGT CAT G
oOR1333 ⁻	5' CCG TGT TGG GTG TAG ATG GGC TCT G

The number given in each primer refers to the position of the 5' base. Numbers given in parentheses for δ OR primers denote corresponding position of the 5' base in the oOR sequence. The +/− symbol indicates sense/antisense polarity of primers relative to mRNA.

cDNA sample to ensure that reverse transcription had occurred. PCR products were analyzed on TBE (0.09 M Tris-borate, 1 mM EDTA, pH 8.0), 2% agarose gels. For restriction analysis with *Bsu*36I, oOR114/oOR925⁻ PCR products were purified with PCR Magic Miniprep columns[®] (Promega Corp.) and eluted in water. PCR products were digested for 2 h at 37°C with 28 U of *Bsu*36I (Promega Corp.), and electrophoresed on a 2% agarose gel.

Isolation of larger PCR products was done by nested PCR to minimize amplification of extraneous products. Nested PCR was performed by first amplifying for 15 cycles of 94°C (1'15") → 57°C (1'15") → 72°C (1'45") with 2 ng of each outer primer (i.e. oOR52⁺ and oOR1333⁻), and then adding 100 ng of each inner primer (i.e. oOR114⁺ and oOR1292⁻) and amplifying for another 30 cycles of 94°C (1'15") → 57°C (1'15") → 72°C (1'15").

oOR PCR products were ligated into TA cloning vectors (Invitrogen Corp., San Diego, CA). Transformed *Escherichia coli* were selected on Luria-Bertani carbenicillin (50 µg/ml) agar plates, coated with X-Gal to allow blue/white selection of recombinant clones. Using oOR primers, PCR was performed on an inoculum of each bacterial colony to identify those bearing oOR plasmid clones. Plasmid DNA was obtained from recombinant clones by the alkaline lysis method (Sambrook et al., 1982) and used directly as a template for sequencing. DNA sequencing was done by the Sanger method (Sanger et al., 1977) with a CircumVent sequencing kit (New England Biolabs Inc., Beverly, MA). Sequencing reaction products were labeled by incorporation of [α -³⁵S]dATP (1000 Ci/mmol; Amersham, Arlington Heights, IL). Identification of DNA sequences and comparison to other ORs was achieved by submission of query sequences to BLAST, an algorithm for identifying homologous regions of sequence (Altschul et al., 1990), at the National Center for Biotechnology Information.

2.5. Northern blot analysis

Analysis of RNA was done on 1–1.2% formaldehyde agarose gels according to the procedure of Sambrook et al. (1982). RNA was blotted onto Nylon membranes (Tropilon, Bedford, MA) with a vacuum blotter (Bio-Rad, Richmond, CA). Blots were irradiated with 200 mJ/cm² in a UV crosslinker (Fisher Scientific, Houston, TX). An 842-bp oOR DNA probe was synthesized by PCR amplification of cloned oOR sequence with δ OR395⁺ and oOR1292⁻. Unincorporated dNTPs were removed from the PCR product by column chromatography (ChromaSpin-100 column, Clontech, Palo Alto, CA), which was then radiolabeled with [α -³²P]dCTP (3000 Ci/mmol; Amersham) by nick translation (Promega Corp.).

Hybridization of radiolabeled probe to Northern blots was achieved at 55–60°C for 12–14 h (55°C for total RNA/60°C for poly(A)⁺ RNA) while shaking in hybridization solution (50 ng labeled probe/ml, 30% formamide, 7% SDS, 120 mM NaH₂PO₄, 250 mM NaCl). Excess probe was removed from membranes by sequential 15-min washes in solutions of 2 × SSC/0.5% SDS, 0.5 × SSC/0.5% SDS, and twice in 0.1 × SSC/0.5% SDS. A final wash was performed at 55–60°C for 10 min in a 0.1 × SSC/0.5% SDS solution.

2.6. Southern blot analysis

Following electrophoresis, PCR products were vacuum-blotted and immobilized on Nylon membranes. Probe synthesis was performed as described above. The FACS lymphocyte RT-PCR products were hybridized with radiolabeled δ OR395⁺/oOR925⁻ PCR product amplified from cloned oOR sequence. The *Bsu*36I-digested RT-PCR products were hybridized with radiolabeled oOR114⁺/oOR925⁻ PCR product amplified from cloned oOR sequence. Probe hybridization and membrane washes were performed as described above.

2.7. Antisense oligonucleotide experiments

Antisense oligonucleotide was prepared against the oOR 5' untranslated region (i.e. oOR153⁻, 5'-AGC-CACTCAGTACAGTTC-3'), and a scrambled sequence of oOR153⁻ (5'-ATCCCTTAATCGCGCAA-G-3') was generated to control for non-specific inhibition. These oligonucleotides were synthesized by LSUMC Core Laboratories.

Proliferation assays were performed, as follows: 5 × 10⁵ splenic lymphocytes in 100 µl complete medium (RPMI 1640 containing 10% fetal bovine serum and 2.5% Hybri-Max (Sigma Chemical Company, St. Louis, MO) antibiotic/antimycotic solution) were placed in

96-well microtiter plates (Costar, Cambridge, MA). 100 μ l complete medium containing lipopolysaccharide (LPS, 1.0 μ g/well) was added to each well, along with 1.0 μ g of antisense (oOR153⁻) or scrambled oligonucleotide. Cells were cultured at 37°C/5% CO₂ for 48 h. [³H]Thymidine (500 nCi) in 10 μ l of Hank's balanced salt solution was added to each well, and the cells were cultured 16–24 h. Cells were harvested on glass fiber filter strips using a multi-well harvester (Cambridge Technologies, Watertown, MA). Filters were placed in scintillation vials containing 6.0 ml of Cytosint liquid scintillation cocktail (ICN, Irvine, CA) and allowed to equilibrate for 2–4 h. The incorporation of [³H]thymidine was determined by liquid scintillation counting using a Beckman LS9800. The mitogenic response of each treatment group was assayed in quadruplicate. Incorporation of [³H]thymidine by cells cultured in the absence of LPS was less than 20% of that obtained in maximally stimulated cultures.

Antibody production from lymphocytes was determined, as follows: 2 \times 10⁶ splenic lymphocytes in 1.0 ml of complete medium were added to 24-well culture plates (Costar). Antisense (oOR153⁻) or scrambled oligonucleotide was added to each well (5.0 or 2.5 μ g) in 5 μ l of phosphate-buffered saline, along with 2.0 μ g of LPS. After 5 days incubation at 37°C/5% CO₂, supernates were harvested and assayed for polyclonal IgG or IgM by ELISA as previously described (Carr et al., 1990b).

3. Results

3.1. Screening lymphocytes for expression of OR mRNA

Splenic lymphocytes were screened for the expression of OR-encoding mRNA transcripts by RT-PCR. Initially, oligonucleotide primers δ OR395⁺ and δ OR695⁻ were made against the coding sequence of TM III and TM V of the δ OR clone, DOR-1 (Evans et al., 1992). These primer sequences were chosen for screening lymphocytes because they are highly conserved among ORs. δ OR395⁺ is 83% and 70% homologous to the TM III-encoding sequence of rat κ - and μ - ORs, while δ OR695⁻ is 84% and 80% homologous, respectively, to the TM V coding sequence of these ORs.

RT-PCR amplification of lymphocyte RNA using the primers δ OR395⁺ and δ OR695⁻ yielded two major populations of PCR products. One PCR product corresponded in size to the predicted 301-bp DOR-1 product, while the other PCR product was approximately 80 bp larger. Upon Southern blotting, however, these lymphocyte-derived PCR products failed to anneal with a DOR-1-specific oligonucleotide probe (not shown). The PCR products were cloned and se-

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      (δOR395+) →
532  GcTgtGctCTcCctTgactactacaacatgtttaccagcactttcoact
580  ttgactgccatgagtgttagaccgttatgttagctatctgccaccctatccg
630  tgcccttgatgttcggacatccagtaaagccagccgcttaattgtggcca
680  tatgggcccctggcttcggtggttggtgttcctgttgccatcatgggctca
730  gcacaagtggaggatgaag|gtcagtgggc agtctctctc

      cctgaccaat cagttcccca tggttcttgc cggcccctct
      gacctcattt ctctctgca g|agatcgagtgcctggtggagatccccgcccc

      ← (δOR695-)
780  tcaggactattggggccctgtatttgccatctgcGtGttcctCtttGccttcg

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Fig. 1. Nucleotide sequence of lymphocyte-derived oOR RT-PCR product obtained with δ OR primers. The sequence of δ OR395⁺ and the complement of δ OR695⁻ are shown in boldface type. δ OR primer bases that were not homologous with oOR are shown in uppercase letters. Bases are numbered according to their position in a mouse oOR cDNA clone. The 81-base intron which was present in the 382-bp oOR PCR product is shown in italics.

quenced, and the 301-bp PCR product was found to be identical in sequence to bases 532 to 832 of an oOR clone isolated from a mouse brain cDNA library (Genbank accession number U04952). The 382-bp PCR product was also amplified from oOR mRNA, but it contained an additional 81 bases of sequence which lie between bases 748 and 749 of the oOR cDNA sequence (Fig. 1). These 81 bases formed an intron motif with 5' splice donor (AAG[↓]GTCAGT) and 3' splice acceptor (CTCTCCTGCAG[↓]) sequences.

Using the same primers, δ OR395⁺ and δ OR695⁻, expression of oOR transcripts in brain and lymphocyte RNA was compared by RT-PCR (Fig. 2). Upon gel electrophoresis, the brain-derived PCR products (lane

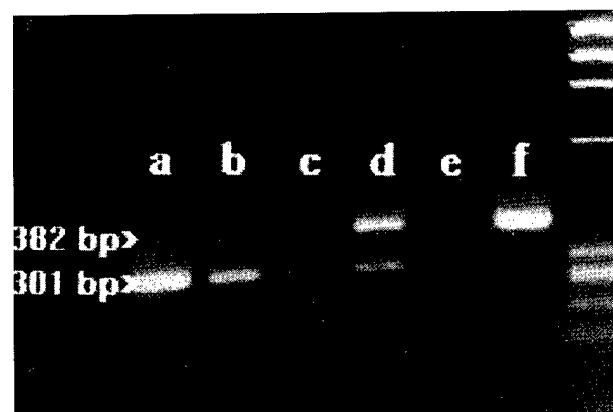


Fig. 2. Detection of oOR expression in brain and lymphocytes by RT-PCR analysis. RT-PCR was performed on RNA samples isolated from brain (lane a), whole spleen (lane b), unstimulated lymphocytes (lane c), and ConA-stimulated lymphocytes (lane d). No template (lane e) and cloned oOR sequence (lane f) served as negative and positive controls, respectively, of PCR amplification. ϕ X174/*Hae*III markers are shown on the far right.

Expression of oOR mRNA in splenic lymphocytes and brain was compared by Northern blot analysis. Two species of RNA transcripts, 3.0 and 1.5 kb, were readily detected in total brain RNA (data not shown). Despite the intense signal from the 3.0-kb transcripts

found in brain RNA, the 1.5-kb band correlates more closely in size to the 1338-base oOR cDNA clone isolated from mouse brain (Genbank accession number U04952), and the 1.3–1.8-kb cDNA clones isolated from rat brain (Bunzow and Grandy, 1994; Chen et al., 1994; Fukuda et al., 1994). Of the spleen-derived total RNA samples, ConA-stimulated but not unstimulated lymphocytes contained a detectable 1.5-kb oOR transcript. Hybridization of a G3PDH (i.e. housekeeping gene) probe to total RNA showed that less G3PDH mRNA was present in unstimulated lymphocytes than in brain and ConA-stimulated lymphocytes (data not shown), which presumably reflects the fact that lymphocytes are a relatively quiescent cell population in vivo, and are less transcriptionally active than brain and mitogen-stimulated lymphocytes.

Northern blot analysis of poly(A)⁺ RNA (Fig. 4A) showed that 1.5-kb oOR mRNA was detectable in both unstimulated (lane b) and ConA-stimulated (lane c)

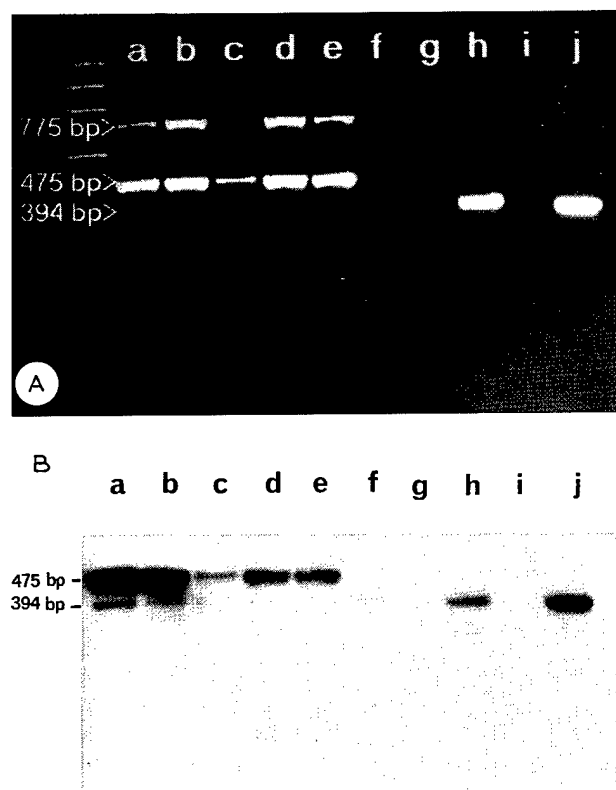


Fig. 5. Detection of oOR expression in CD4⁺, CD8⁺, and CD4⁻CD8⁻ lymphocytes by RT-PCR analysis. (A) Agarose gel analysis of oOR395⁺/oOR925⁻ RT-PCR products. RNA samples were isolated from cell-sorted CD4⁺, CD8⁺, CD4⁻CD8⁻ lymphocytes (lanes a, b, c); total cells prior to sorting (lane d), Lympholyte M[®]-purified lymphocytes (lane e), EL4 cells (lane f), L929 cells (lane g), and brain (lane h). No template (lane i) and cloned oOR sequence (lane j) served as negative and positive controls, respectively, of PCR amplification. ϕ X174/*Hae*III markers are shown on the far left. (B) Southern blot of the above gel hybridized with oOR probe.

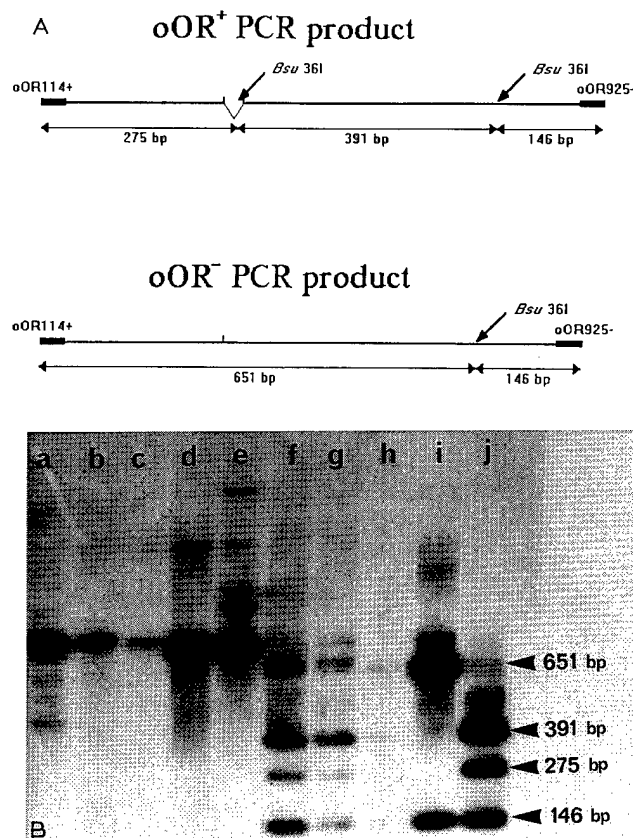


Fig. 6. *Bsu*36I restriction analysis of oOR RT-PCR products. (A) *Bsu*36I restriction map of oOR114⁺/oOR925⁻ RT-PCR products amplified from oOR⁺ and oOR⁻ mRNA. (B) The Southern blot shown contains untreated oOR114⁺/oOR925⁻ PCR products (lanes a–e), and aliquots of the same samples following digestion with *Bsu*36I (lanes f–j). These RT-PCR products were amplified from brain poly(A)⁺ RNA (lanes a and f), brain total RNA (lanes b and g), lymphocyte total RNA (lanes c and h). The standards for the *Bsu*36I restriction digests were PCR products amplified from pLoOR⁻ (lanes d and i), and pLoOR⁺ (lanes e and j). A control PCR tube without template did not yield any products (not shown).

splenic lymphocytes. A lighter exposure of the same Northern blot (Fig. 4B) shows that brain poly(A)⁺ RNA contained three predominant mRNA species (1.5, 3.0, and 4.0 kb) that hybridized with oOR probe. Based on phosphorimager densitometric analysis, the unstimulated and ConA-stimulated splenic lymphocyte poly(A)⁺ RNA samples contained 4.5% and 22.5% as much 1.5 kb oOR mRNA as was found in the brain.

3.4. Expression of oOR mRNA in lymphocyte subpopulations

In order to assess oOR mRNA expression in lymphocyte subpopulations, RT-PCR was performed using RNA from CD4⁺, CD8⁺, and CD4⁻CD8⁻ cell-sorted lymphocytes (Fig. 5A). A Southern blot of the resulting PCR products is shown in Fig. 5B. Both brain RNA (lane h) and pLoOR⁻ (lane j) yielded a PCR product

of the predicted 394-bp size. The majority of oOR PCR products obtained from the lymphocyte-derived RNA samples (lanes a–c), however, were 475 bp, indicating that the 81-base intron motif was present. A non-oOR PCR product (i.e. one that did not hybridize with the oOR probe) of approximately 775 bp was also amplified from CD4⁺, CD8⁺, and total lymphocyte RNA (lanes a, b, and d–e), but not from CD4[−]CD8[−] lymphocytes nor any of the other RNA samples (Fig. 5A). Based on RT-PCR analysis (lanes a–c), oOR mRNA appears to be expressed in all three lymphocyte subpopulations.

3.5. Restriction analysis of oOR RT-PCR products

The absence of a 15-base intron motif (bases 378–392) in pLoOR[−] suggested that alternative splicing of oOR mRNA occurs in lymphocytes. Because a *Bsu*36I restriction site (CCTNAGG, bases 387–393) occurs in the intron, RT-PCR products amplified from oOR mRNA lacking bases 378–392 did not contain the restriction site. Therefore, *Bsu*36I restriction analysis provided a method by which RT-PCR products amplified from oOR⁺ mRNA (bases 378–392 present) could be differentiated from RT-PCR products amplified from oOR[−] mRNA (bases 378–392 absent). Restriction

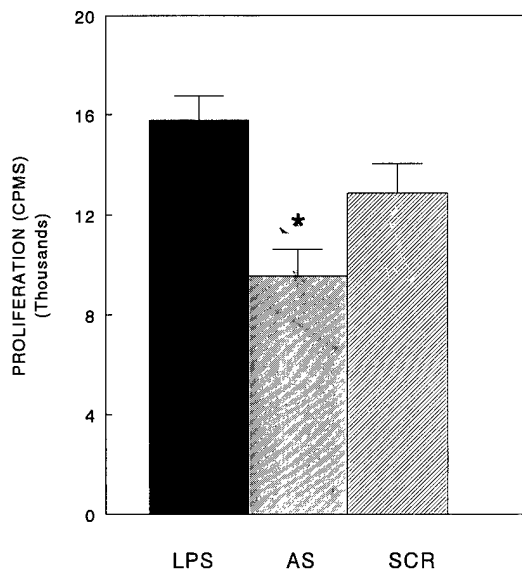


Fig. 7. Orphan OR antisense oligonucleotide blocks LPS-induced lymphocyte proliferation. Splenic lymphocytes (5×10^5 cells) were stimulated with LPS in the presence or absence of antisense OR oligo (AS, 1.0 μ g) or scrambled oligo (SCR, 1.0 μ g). Cells were then assessed for proliferation by [³H]thymidine uptake 72 h following initiation of culture. Bars represent S.E.M. This figure represents the summary of three independent experiments with each condition measured in quadruplicate/experiment. * $P < 0.05$, $F(2,8) = 8.4459$ comparing the oligo-treated cultures to LPS only culture as determined by ANOVA and Scheffé multiple comparison test.

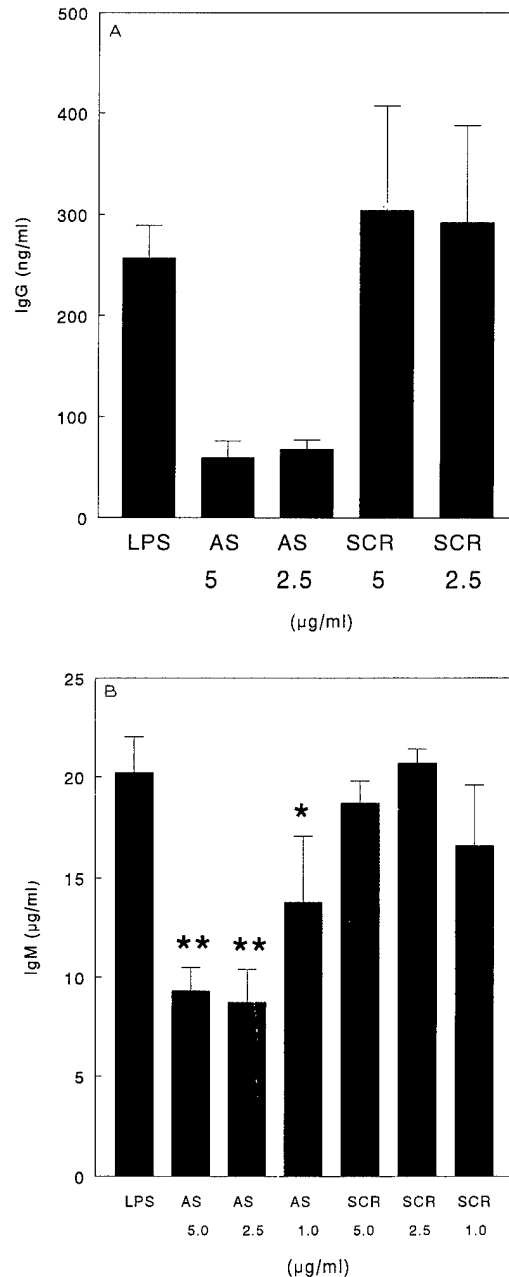


Fig. 8. Orphan OR antisense oligonucleotide blocks LPS-induced polyclonal antibody production. Splenic lymphocytes (2×10^6 cells) were stimulated with LPS in the presence or absence of the indicated concentration of antisense (AS) or scrambled (SCR) oligos. Supernates were collected 5 days following the initiation of culture and assayed for polyclonal IgG (A) or polyclonal IgM (B). Bars represent S.E.M. The figures are a summary of three independent experiments with each condition measured in triplicate/experiment. * $P < 0.05$, ** $P < 0.01$, $F(6,20) = 6.1263$ as determined by ANOVA and Tukey's post *t*-test comparing the oligo-treated cultures to the LPS-only culture.

tion maps of oOR⁺ and oOR[−] PCR products obtained in these experiments are shown in Fig. 6A.

In Fig. 6B, a radiolabeled oOR probe was hybridized to untreated oOR114⁺/oOR925[−] PCR prod-

[illegible]

ucts (lanes a-e) and *Bsu*36I-digested oOR114⁺/oOR925⁻ PCR products (lanes f-j). The untreated PCR products (lanes a-e) all migrated at a rate corresponding to the predicted size of 812 bp. All oOR114⁺/oOR925⁻ PCR products treated with *Bsu*36I were cleaved into 146-bp (lanes f-j) and either 651-bp or 666-bp fragments because of a restriction site which occurs at bases 778-784. The 651-bp PCR product fragments derived from pLoOR⁻ (lane i) and oOR⁻ mRNA (lanes f-h) lacked the intron and were not further cleaved by *Bsu*36I. However, the 666-bp PCR product fragments amplified from pLoOR⁺ (lane j) and oOR⁺ mRNA (lanes f-h) contained the intron, and were thus cut into 275-bp and 391-bp fragments by *Bsu*36I.

3.6. Antisense oligonucleotide defines a functional role for lymphocyte oOR

Lymphocytes have been reported to secrete endorphins following LPS stimulation (Harbour-McMenamin et al., 1985). To determine the potential relationship between lymphocyte-derived endorphins and orphan opioid receptors on immune function, antisense oligonucleotides were used to block oOR expression. Lymphocytes were stimulated with LPS in the presence of either oOR153⁻ (oOR-antisense oligonucleotide) or a scrambled control sequence, and their effects on proliferation and antibody production were compared. Orphan OR153⁻ significantly reduced the LPS-stimulated proliferation of splenic lymphocytes by $49.9 \pm 1.7\%$ (Fig. 7). By comparison, the scrambled oligonucleotide inhibited LPS-induced proliferation as well ($22 \pm 8.0\%$) (Fig. 7). Similarly, oOR153⁻ significantly inhibited (50–75%) polyclonal IgG and IgM production from LPS-stimulated splenic lymphocytes, but the scrambled oligonucleotide had no effect (Fig. 8).

3.7. Nucleotide sequence

This sequence has been deposited in the Genbank database under accession number U14165.

4. Discussion

There is evidence that δ ORs are present on lymphocytes (Carr, 1991), but this has not been confirmed at the level of gene expression. To address this point, we screened lymphocyte RNA for the presence of

δ OR-encoding RNA transcripts by RT-PCR. δ OR mRNA was not detected, but rather the selected primer pair detected expression of oOR mRNA. Overall, oOR shares 61% amino acid identity with δ OR and 58% with μ - and κ - ORs, but sequence conservation amongst the ORs is particularly high within the TM spanning regions (Fig. 9). When the comparison of sequence homology is extended to analogous amino acids, oOR shares 73%, 74%, and 76% similarity with μ -, κ -, and δ -ORs, respectively.

Functional expression of the human oOR cDNA clone, hORL1, in COS cells showed that oOR did not bind endogenous opioid ligands. However, the universal opiate agonist etorphine did bind and cause suppression of adenylate cyclase activity in COS cells, and these effects were sensitive to diprenorphine, an opioid antagonist (Mollereau et al., 1994). In situ hybridization indicates that differential expression of oOR occurs in rat brainstem (Fukuda et al., 1994), suggesting a role for oOR in modulation of neurotransmission in the neuroendocrine system.

Exogenous (Bussiere et al., 1993) and endogenous (for review, Carr, 1991) opioids have been shown to modulate antibody production. LPS has been shown to induce leukocyte production of endogenous opioids and the endorphins (Harbour-McMenamin et al., 1985). In the present study, oOR-specific antisense oligonucleotides blocked antibody production from splenic lymphocytes, and had a modest effect on proliferation. The results suggest that oORs play a role in autocrine regulation of lymphocyte function. Therefore, lymphocyte oORs are potentially significant sites of immunoregulation.

Post-transcriptional processing of oOR RNA transcripts appears to be different in lymphocytes than in brain. RT-PCR of brain RNA with δ OR395⁺ and oOR925⁻ yields a 394-bp PCR product, while lymphocyte RNA yields 475-bp and 394-bp oOR-derived PCR products. The difference in size presumably reflects the presence (475 bp) or absence (394 bp) of an 81-base intron in the original oOR RNA template. Therefore, while brain-derived oOR transcripts are fully processed, an 81-base intron is present in a large fraction of lymphocyte-derived oOR transcripts. Given that the extra 81 bases would not disrupt the open reading frame, this RNA species could have encoded an oOR subclass with an additional 27 amino acids in the second extracellular loop. Translation of the 81-base sequence, however, revealed that a UGA stop codon was present (verified in three independent clones), making it unlikely that this oOR RNA species encodes a functional oOR. The existence of this immature oOR RNA species suggests that lymphocyte expression of oOR may be regulated post-transcriptionally at the level of mRNA splicing.

Sequence analysis of two oOR114⁺/oOR1292⁻

PCR products revealed that a 15-base intron motif (bases 378–392) which is present in all identified oOR cDNA clones (Bunzow and Grandy, 1994; Chen et al., 1994; Fukuda et al., 1994; Mollereau et al., 1994) was spliced from pLoOR⁻. *Bsu*36I restriction analysis of oOR114⁺/oOR925⁻ RT-PCR products confirmed that alternative splicing of oOR mRNA occurs in brain and lymphocytes. Assuming that RT-PCR did not preferentially amplify one oOR splice variant, phosphorimager densitometric analysis indicated that bases 378–392 are removed from 25–30% of oOR mRNA in brain, and approximately 45% of oOR mRNA in lymphocytes.

Pharmacological characterization indicates that there is more than one type of δ -, κ -, and μ -OR. Likewise, alternative splicing of bases 378–392 of oOR mRNA which encode Y⁷¹-R⁷⁵ suggests that at least two functionally distinct oOR subtypes exist. For example, removal of Y⁷¹-R⁷⁵ from the first intracellular loop may sterically alter oOR, thereby changing the ligand affinity of this receptor subtype. Likewise, removal of a potential tyrosine kinase substrate (i.e. YXX[L/I]) are phosphorylation sites in antigen recognition activation motifs found in a variety of lymphocyte-derived receptors, e.g. the CD3 ϵ and γ chains) could produce an oOR subtype that is differentially regulated.

Northern blot analysis confirmed that a 1.5-kb oOR mRNA transcript is expressed in both stimulated and unstimulated murine splenic lymphocytes. Because poly(A)⁺ RNA was isolated from unfractionated spleen cells, oOR mRNA could have potentially been derived from non-lymphoid cells in the spleen. However, the observed induction of oOR mRNA expression by ConA suggests that lymphocytes are the major source of oOR mRNA in the poly(A)⁺ RNA blot. Furthermore, this hypothesis is supported by RT-PCR analysis of FACS-purified spleen cells which detected oOR mRNA in cells bearing CD4⁺ and CD8⁺ lymphocyte differentiation markers.

In conclusion, a gene encoding an opioid-like receptor is expressed in mouse lymphocytes. This finding lends support to pharmacological studies that have identified opioid binding sites on lymphocytes (Madden et al., 1987; Carr et al., 1989; Ovadia et al., 1989; Bidlack et al., 1992), as well as in vitro results which indicate that endogenous opioids modulate lymphocyte function (Johnson et al., 1982; Mathews et al., 1983; Van Epps and Saland, 1984; Heijnen et al., 1986; Carr and Klimpel, 1986; Mandler et al., 1986; Taub et al., 1991). Lymphocyte expression of neuropeptide receptors has been proposed as a mechanism by which neuroendocrine regulation of immune function occurs (Carr, 1991). Having identified and sequenced an opioid-like receptor mRNA in lymphocytes, further studies can begin to explore the function of oOR in neuroimmunomodulation.

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Mechanisms of Herpes Simplex Virus Type 1 Reactivation

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Primary cultures of trigeminal ganglion (TG) cells from herpes simplex virus type 1 (HSV-1) latently infected mice were used to study reactivation. Expression of HSV-1 latency-associated transcripts was noted in TG cell cultures. Infectious virus appeared in 75% of culture supernatants within 120 h after heat stress. Likewise, HSV-1 lytic-phase mRNA and proteins were detectable 24 h after heat stress. HSV-1 antigen first appeared in neurons after heat stress, indicating the neurons were the source of reactivation. The effect of heat stress duration on reactivation was determined. Reactivation occurred in 0, 40, or 67% of cultures after a 1-, 2-, or 3-h heat stress, respectively. However, 72-kDa heat shock protein expression was induced regardless of heat stress duration. Thus, reactivation was not a direct result of inducing the heat shock response. The capacities of several drugs to induce reactivation were also evaluated. While neither epinephrine, forskolin, nor a membrane-permeable cyclic AMP analog induced reactivation, dexamethasone did so in a dose-dependent manner. Furthermore, dexamethasone pretreatment enhanced the kinetics of heat stress-induced reactivation from TG cells. Collectively, the results indicate that TG cell cultures mimic important aspects of in vivo latency and reactivation. Therefore, this model may be useful for studying signalling pathways that lead to HSV-1 reactivation.

Despite two decades of research since sensory neurons were identified as the site of latent herpes simplex virus type 1 (HSV-1) infection (6), little is known about the mechanisms by which stress induces reactivation. While our knowledge of the HSV-1 genome and its gene products has increased tremendously, no new clinical treatments have been developed to block reactivation. Stressors such as epinephrine iontophoresis (19), cyclophosphamide and dexamethasone (Dex) administration (7), UV irradiation (20), and transient hyperthermia (35) induce reactivation in animal models. In no model, however, are the events that occur between stress and production of infectious HSV-1 known. Therefore, many unresolved questions remain. What are the reactivation-inducing signals that impinge upon latently infected neurons? Through what receptors are those signals recognized and transduced across the cell membrane? What intracellular effectors carry these signals to the nucleus? What changes in HSV-1 gene regulation constitute the event referred to as reactivation? Understanding these molecular mechanisms would greatly facilitate the rational development of reactivation-blocking drugs. Because such questions are difficult to address in vivo, an in vitro model which mimics the in vivo situation would provide an important tool for studying mechanisms of reactivation.

Culture systems in which HSV-1 reactivation occurs have been described, but most are poor models of in vivo latency and reactivation. For example, coculture of trigeminal ganglion (TG) explants with HSV-1 permissive cells is a useful assay for verifying latent infection (43) but is of limited value as a reactivation model because reactivation is induced regardless of treatment. Neonatal sensory neuron cultures are a good model for studying reactivation because HSV-1 latency is maintained until administration of a stressor (41, 48). How-

ever, this model differs substantially from adult ganglionic neurons in vivo. All of the cells in the neonatal cell cultures are neurons, and depending on the multiplicity of infection, up to 100% of these neurons can be latently infected. In contrast, 5 to 10% of cells in the ganglion are neurons, and only 5 to 10% of those become latently infected following HSV-1 ocular infection (14).

Moriya et al. (26) described a culture system which is potentially useful as an in vitro model of stress-induced reactivation. In the presence of an antiviral drug, primary cell cultures were established from latently infected mouse TGs. Latency was maintained following removal of the antiviral drug, and the investigators found that HSV-1 could be reactivated from TG cells by heat stress. Because critical points (e.g., demonstration that cytopathic effects were due to HSV-1) were not addressed in this study, the results were suggestive but not compelling.

In the present study, we confirm the results of Moriya et al. (26) and demonstrate that TG cell cultures mimic important aspects of in vivo latency and reactivation. Evidence is provided that (i) neurons remain viable in TG cell cultures, (ii) latency-associated transcription continues in culture, (iii) HSV-1 mRNA, protein, and virion synthesis are efficiently reactivated following heat stress, and (iv) latently infected neurons are the source from which HSV-1 reactivation occurs.

The capacities of cyclic AMP (cAMP), epinephrine, and Dex to induce HSV-1 reactivation from TG cell cultures were also studied. These compounds were chosen, in particular, because of their reactivation-inducing capacity in other herpesvirus models (19, 32, 41) and because of their relationship to the in vivo stress response. While epinephrine and cAMP had no effect on TG cells, Dex induced HSV-1 reactivation in a dose-dependent manner. Furthermore, Dex enhanced the kinetics of heat stress-induced reactivation. The results suggest a central role for glucocorticoid hormones in vivo in stress-induced HSV reactivation.

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TABLE 1. Oligonucleotide primers used in this study

Primer	Location in HSV-1	Sequence	Reference
G3PDH-a		GAATCTACTGGCGTCTTCACC	18
G3PDH-b		GTCAATGAGCCCTTCCACGATGC	
ICP0-3'	←120987	TTCGACACAGGGGACCCCTAGT	22
LAT-a	120702→	GACAGCAAAAATCCCCTGAG	
LAT-b	←120896	ACGAGGGGAAAACAATAAGGG	
ICP27-a	114922→	TTTCTCCAGTGCTAGCTGAAGG	2
ICP27-b	←115204	TCAACTCGCAGACACGACTCG	
RR-17	88517→	ATGCCAGACCTGTTTTTCAA	15
RR-b	←88759	GTCTTTGAACATGACGAAGG	
TK-a	46622→	ATACCGACGATCTGCGACCT	22
TK-b	←46731	TTATTGCCGTCATAGCGCGG	

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MATERIALS AND METHODS

Animals and cells. Female 25- to 34-g ICR mice (Harlan-Sprague Dawley, Indianapolis, Ind.) were used in these experiments. The CV-1 African green monkey kidney cell line was obtained from the American Type Culture Collection (Rockville, Md.).

Infection of mice. Bilateral ocular infection of ICR mice (Harlan-Sprague Dawley) was performed as follows. Corneas were scarified with a 25-g needle, and tear film was blotted from the eyes with tissue. Mice were infected by placing 3 µl of medium containing 10⁵ PFU of HSV-1 (McKrae strain) per ml on each eye. At the time of infection, mice were passively immunized with 0.1 ml of rabbit antiserum to HSV-1 by intraperitoneal injection to enhance survival. To verify primary infection, mouse eyes were swabbed 2 days postinoculation, and swabs were transferred to CV-1 monolayer cultures, which were observed for HSV-1-induced cytopathic effects.

Establishment of TG cell cultures. TG cell cultures were prepared according to a modified version of the protocol described by Moriya et al. (26). TG cells were cultured in minimum essential medium containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, Md.), antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, Mo.), and 10 ng of nerve growth factor 2.5s (Collaborative Biomedical Products, Bedford, Mass.) per ml (TG medium). TGs were aseptically removed from latently infected mice (greater than 28 days postinoculation) and placed in TG medium on ice. TGs were pooled in 1.5 ml of calcium- and magnesium-free Hank's balanced saline solution containing collagenase type XI (1 mg/ml; Sigma) and collagenase type IV (1 mg/ml; Sigma) and incubated at 37°C for 75 to 90 min. To facilitate dissociation, ganglia were triturated every 25 min with a 1-ml serological pipette.

Next, 8.5 ml of TG medium was added to the dissociated cells, which were then pelleted by centrifugation (4°C, 5 min, 200 × g), and the collagenase-containing supernatant was discarded. The cells were rinsed twice more with TG medium and resuspended in 50 ml of TG medium containing 5 µg/ml of (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU; Sigma) per ml. The cells were distributed into two 24-well culture plates (1 ml of cell suspension per well) which had been thin coated with rat tail collagen type I (50 µg/ml; Collaborative Biomedical Products) and recombinant mouse laminin (2.5 µg/ml; Collaborative Biomedical Products) according to the vendor's directions. To promote cell adherence, culture plates were centrifuged for 3 min at 200 × g.

Cultures were incubated in a 37°C tissue culture incubator (5% CO₂, 95% humidity). Two days after culture establishment, 0.5 ml of TG medium was added to each culture well. Five days after culture establishment, BVDU was removed by replacing the culture medium. Cultures were incubated for 14 to 16 days before initiation of reactivation experiments. Culture medium was partially replaced every 5 to 7 days.

Heat stress and drug treatments. TG cells were heat stressed by placing culture plates in a 43°C tissue culture incubator (5% CO₂, 95% humidity) for 3 h.

In drug experiments, cultures were incubated with either forskolin for 1 h, chlorophenylthio (CPT)-cAMP for 1.5 h, epinephrine for 1.5 h, or Dex for the duration of the experiment. Five days after drug treatment, cultures were heat stressed to verify the presence of latent HSV-1.

The effect of forskolin on intracellular cAMP concentrations was determined 30 min after addition of forskolin to TG cells, using a cAMP enzyme-linked immunosorbent assay (PerSeptive Diagnostics, Cambridge, Mass.).

Monitoring of TG cultures for HSV-1 reactivation. Infectious HSV-1 was detected in cultures by transferring 100 µl of culture medium to CV-1 cell monolayers in 96-well plates and monitoring CV-1 cells for the appearance of cytopathic effect. Each TG culture well was sampled for infectious virus at 4, 8, and 12 days after plating and immediately before administration of a potential

reactivation-inducing stimulus. Thereafter, wells were sampled every 24 h for 5 days to record the appearance of infectious virus.

Reverse transcription-PCR (RT-PCR). Total RNA was extracted directly from culture wells with Ultraspec RNA isolation reagent (Biotecx Inc., Houston, Tex.) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on equivalent amounts of RNA from each sample, using an oligo(dT)₁₅ primer and avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, Wis.). Because the major species of latency-associated transcripts (LAT) is not polyadenylated (10), reverse transcription of LAT was achieved by using a LAT-specific primer, ICP0-3'. PCR was performed on equivalent amounts of cDNA; each reaction mixture contained 1× Taq buffer, 0.25 µM each PCR primer, 100 µM each deoxynucleoside triphosphate, and 2.5 U of Taq polymerase (Promega), and each reaction was performed in an MJ Research (Watertown, Mass.) thermal cycler with 35 cycles of 94°C (1 min 15 s) → 57°C (1 min 15 s) → 72°C (30 s). Oligonucleotide primers were obtained from LSU Medical Center Core Laboratories (New Orleans, La.) and are listed in Table 1. Densitometry of ethidium bromide-stained agarose gels was performed with an EagleEye II still video system (Stratagene, La Jolla, Calif.).

Immunocytochemical staining. Cell cultures were fixed with 10% formalin and treated for 10 min with 0.1 N sodium azide containing 0.3% H₂O₂ to inactivate endogenous peroxidases. HSV-1 antigen was identified by using a rabbit anti-HSV-1-horseradish peroxidase conjugate (DAKO Corporation, Carpinteria, Calif.). Neurons were labeled with rabbit anti-neuron-specific enolase (Zymed Laboratories Inc., San Francisco, Calif.), and the primary antibody was detected with a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, Calif.). Antibody labeling was visualized with the substrate aminoethylcarbazole (Vector Laboratories).

Western blot (immunoblot) analysis. Following lysis of cultured cells in non-denaturing gel loading buffer, debris was pelleted by centrifugation. Aliquots of clarified supernatant were loaded on sodium dodecyl sulfate-7.5% polyacrylamide minigels. Separated proteins were electroblotted onto nitrocellulose (BioBlot NC; Corning Costar, Cambridge, Mass.) and fixed in methanol-Tris buffer. A mouse anti-72/73-kDa heat shock protein monoclonal antibody (Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada) was used to detect 72-kDa heat shock protein HSP-72, which was visualized with the Western Light chemiluminescence detection system (Tropix Inc., Bedford, Mass.).

RESULTS

Neurons in TG cell cultures. Nerve fibers are the conduit by which HSV-1 enters the ganglion during acute infection, and neurons are the only known reservoir of viral DNA during latency (24, 30, 45). Therefore, we sought to verify that neurons survive ganglion dissociation and remain viable in TG cell cultures. Neurons were identified by light microscopy (Fig. 1A) on the basis of their large size and distinct nucleus. Immunocytochemical staining for neuron-specific enolase showed that these cells were in fact neurons (Fig. 1B). The number of neuron-specific enolase-positive cells per culture well was determined to be 410 ± 48 (mean ± standard deviation; n = 5 wells). Because each well received 0.4 ganglion equivalent of cells, approximately 1,000 neurons per ganglion survived dissociation and became adherent in culture.

Latency-associated transcription. During latent infection of the TG in vivo, HSV-1 LATs are detected exclusively in latently infected neurons by in situ hybridization (42, 44). Because TGs were taken from latently infected mice (greater than 28 days postinoculation), RT-PCR analysis was performed to determine if LAT expression persisted in TG cells following dissociation. LATs were detected in 10 of 10 cultures 14 days after establishment (Fig. 2). Presumably, latently infected neurons which survived dissociation are the source of LAT expression in TG cell cultures. However, in situ hybridization will be required to formally address this point.

Effect of BVDU in establishment of TG cell cultures. TG cells were initially cultured in the presence of an antiviral drug, BVDU (9), to prevent reactivation following ganglion dissociation. Contrary to previous results (26), reactivation did not always occur from TG cells established in the absence of antiviral drug. Reactivation occurred in 57% (16 of 28) of cultures established in the absence of BVDU. The lack of reactivation from the other TG cells was not due to an absence of

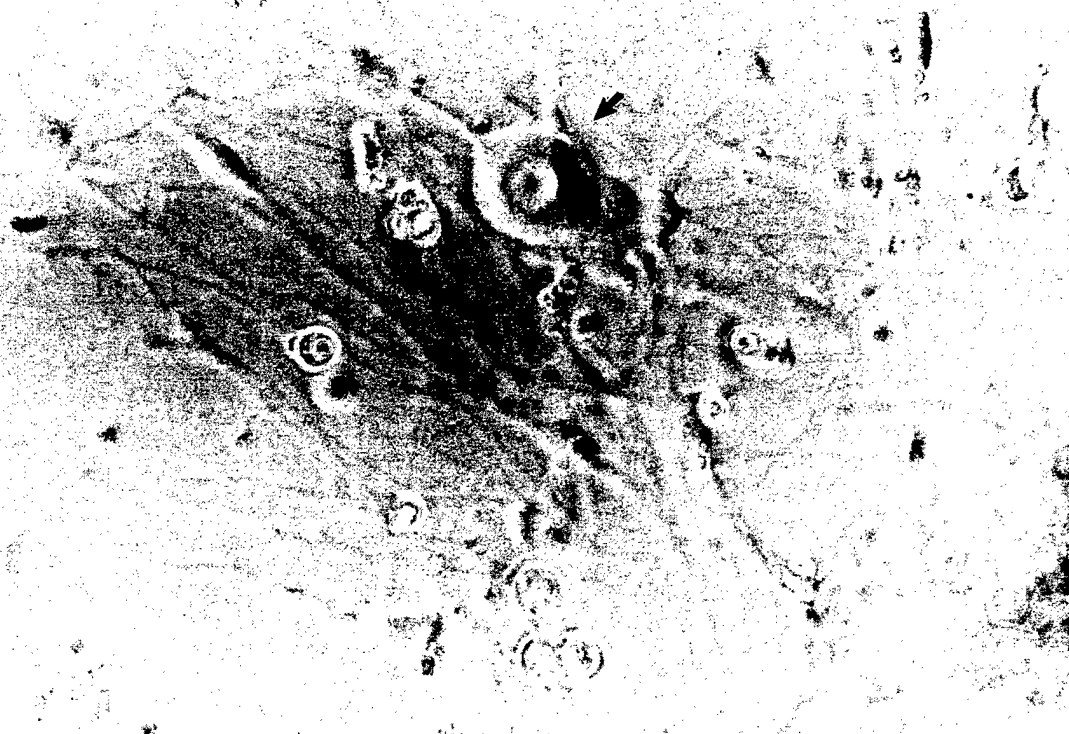
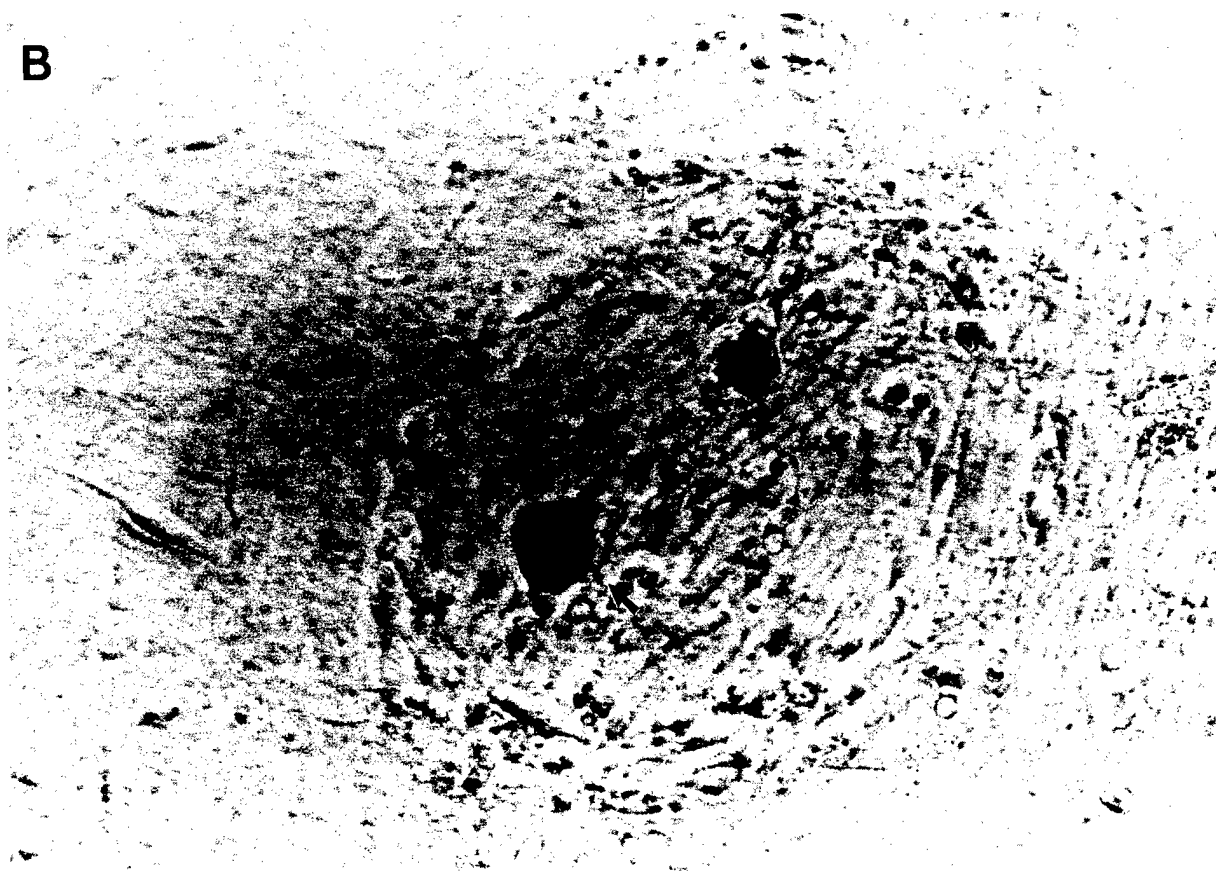
A**B**

FIG. 1. Neurons in TG cell cultures. (A) Phase-contrast appearance of a neuron in culture (indicated by arrow) (magnification, $\times 40$). Note regenerated neurite extending from left side of neuron. (B) Immunocytochemical staining reveals the presence of neuron-specific enolase (indicated by arrow) (magnification, $\times 20$).



FIG. 2. Agarose gel analysis of LAT RT-PCR products (195 bp) amplified from cell cultures 1 to 10 (lanes 1 to 10). Each RNA sample was derived from the cells in a single culture well. No template (-) and HSV-1 DNA (+) served as negative and positive controls, respectively, in the PCR assay. ϕ X174/HaeIII markers are shown on the far left.

latent HSV-1; reactivation later occurred from 10 of the 12 remaining cultures following heat stress.

To determine the drug concentration which would consistently prevent HSV-1 reactivation, cultures were established in medium containing half-log dilutions of BVDU ranging from 0.5 to 50 μ g/ml. Reactivation did not occur in cultures containing 1.6 μ g or more of BVDU per ml ($n = 4$ per group).

RT-PCR analysis of HSV-1 gene expression was performed to verify the efficacy of 5 μ g of BVDU per ml in preventing HSV-1 reactivation during culture establishment. Infected cell polypeptide 27 (ICP27) mRNA was present 24 h after culture establishment but was not detectable by 120 h (Fig. 3A). Therefore, HSV-1 immediate-early gene expression is only transiently upregulated in BVDU-treated cultures. In contrast, expression of HSV-1 ICP27 mRNA increased steadily after culture establishment in the absence of BVDU (Fig. 3A). While LAT expression was detected at all times in BVDU-treated cultures, LAT expression was downregulated in 3 of 4 untreated cultures during the 24- to 48-h time period when reactivation was first occurring (Fig. 3A). PCR of TG cell culture DNA verified that 5 μ g of BVDU per ml effectively prevents HSV-1 DNA replication following culture establish-

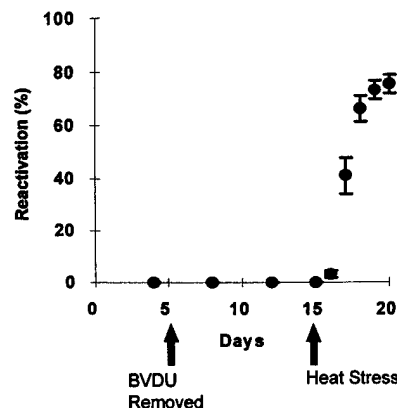


FIG. 4. Kinetics of heat stress-induced HSV-1 reactivation. Heat stress was administered 15 days after culture establishment. Reactivation is defined as the appearance of infectious virus in culture supernatants. Datum points represent the mean frequencies of reactivation (\pm standard errors of the means) observed in 11 experiments.

ment. Whereas amplification of HSV-1 DNA in untreated cultures was evident by 48 and 120 h after culture establishment, HSV-1 DNA levels remained constant in BVDU-treated cultures (Fig. 3B).

Heat stress-induced HSV-1 reactivation. Reactivation did not occur in any cultures following removal of BVDU from cells (5 days after culture establishment). However, heat stress rapidly induced HSV-1 reactivation (Fig. 4) from $74.7\% \pm 3.5\%$ of TG cell cultures ($n = 257$ wells). This finding is consistent with the results of Moriya et al. (26). Of the cultures which eventually reactivated, HSV-1 was detectable in 88% of culture supernatants within 72 h after heat stress. In contrast, reactivation occurred from 0% of nonstressed controls ($n = 52$) over a 20-day culture period.

Heat stress induction of HSV-1 gene expression. In most experiments, reactivation was defined as the appearance of infectious virus in culture supernatants which caused cytopathic effect when virus was transferred to CV-1 cell monolayers. To confirm that heat stress triggered HSV-1 reactivation from TG cells, heat stress induction of ICP27 mRNA expression was verified by RT-PCR (Fig. 5). While ICP27 mRNA was not detectable before or 12 h after the initiation of heat stress, ICP27 mRNA was evident in TG cell cultures by 24 and 48 h after heat stress. In contrast, glyceraldehyde-3-phosphodehydrogenase (G3PDH) mRNA was uniformly present in all RNA samples. After normalization for G3PDH, densitometry indicated that the amount of ICP27 PCR product amplified from TG cell RNA samples increased 18-fold between 24 and 48 h after heat stress.

Immunocytochemical staining was used to determine the kinetics of HSV-1 protein induction following heat stress. Cultured cells did not express detectable HSV-1 protein prior to

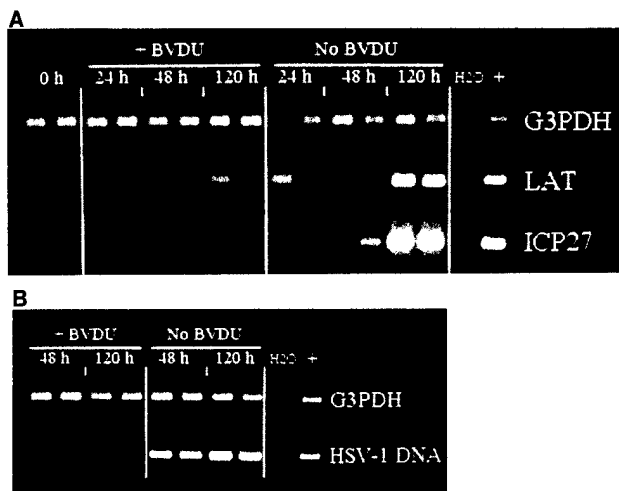


FIG. 3. BVDU treatment prevents HSV-1 reactivation. (A) Effect of BVDU on HSV-1 immediate-early gene expression, determined by RT-PCR analysis of RNA from duplicate samples of TG cells taken 0, 24, 48, and 120 h after establishment of TG cell cultures. The cells were all derived from the same pool of dissociated TGs. + BVDU, cultures that were BVDU treated; No BVDU, cultures that were untreated. RT-PCR was used to detect G3PDH, LAT, and ICP27 mRNAs. Each RNA sample was derived from the pooled cells of three culture wells. (B) Effect of BVDU on HSV-1 DNA replication. PCR was performed on equivalent amounts of DNA from TG cell cultures 48 and 120 h after culture establishment to detect the cellular G3PDH gene and HSV-1 DNA. HSV-1 ribonucleotide reductase primers were used to detect HSV-1 DNA. Each DNA sample was derived from the pooled cells of three culture wells. No template (H₂O) and HSV-1 DNA (+) served as negative and positive controls, respectively, in each PCR assay.

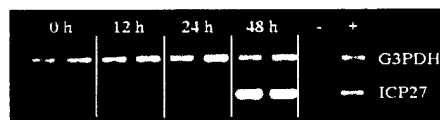


FIG. 5. Kinetics of ICP27 mRNA induction following heat stress. RT-PCR was used to detect G3PDH and HSV-1 ICP27 mRNAs in duplicate samples of TG cells prior to heat stress (0 h) and at 12, 24, and 48 h after initiation of heat stress. No template (-) and HSV-1 DNA (+) served as negative and positive controls, respectively, in each PCR assay. The samples shown are a subset of VEH PCR samples shown in Fig. 10.

heat stress. However, HSV-1 proteins were detected in TG cell cultures 22, 48, and 72 h after heat stress (Fig. 6A to C). 1HSV-1 antigen expression was limited to large, neuron-like cells 22 h after heat stress (Fig. 6D). Large foci of HSV-1 antigen-positive cells were seen 48 and 72 h after heat stress. On the basis of the discrete, circular pattern of staining, each HSV-1 antigen-positive cell cluster appeared to be the result of focal spread from a single reactivation event. The average numbers of reactivation events detected per culture well were 0.5 ($n = 8$ wells), 1.5 ($n = 4$ wells), and 1.75 ($n = 4$ wells) at 22, 48, and 72 h after heat stress, respectively. Therefore, it appears that only a fraction of neurons in which reactivation occurred contained detectable HSV-1 protein by 22 h after heat stress.

Effect of heat stress duration on HSV-1 reactivation. The effect of heat stress duration on TG cell cultures was studied to better characterize the mechanism by which heat stress induces HSV-1 reactivation. TG cells were heat stressed for 1, 2, and 3 h at 43°C and observed for HSV-1 reactivation (Fig. 7, 1st heat stress). Consistent with other experiments, HSV-1 reactivated from 67% (8 of 12) of TG cell cultures heat stressed for 3 h. However, 2 h of heat stress induced reactivation from only 40% of cultures ($n = 28$), and 0% of cultures ($n = 28$) reactivated following 1 h at 43°C. To verify the presence of reactivatable virus, the cultures previously treated for 1, 2, or 3 h were heat stressed a second time for 3 h (Fig. 7, 2nd heat stress). After a 3-h heat stress, HSV-1 reactivated from 61% of cultures previously heat stressed for 1 h. Likewise, the frequency of reactivation increased from 40 to 61% in those cultures previously heat stressed for 2 h. Interestingly, the frequency of reactivation increased from 67 to 92% in cultures that had been heat stressed for 3 h the first time. Twelve TG cell cultures that were not heat treated did not reactivate over the 25-day course of this experiment.

The results of others suggest that HSV-1 reactivation may be triggered by induction of the cellular heat shock response (26, 35). Expression of HSP-72 (often used as a marker of heat shock) was rapidly induced in TG cells following transfer to a 43°C incubator. While HSP-72 was not detected before or 1 h after transfer to 43°C, HSP-72 expression was evident 2 and 3 h after initiation of heat stress (Fig. 8A).

To account for the observed lack of reactivation after 1 h of heat stress, we thought that perhaps 1 h in a 43°C incubator was insufficient to induce a heat shock response. Therefore, the effect of heat stress duration on HSP-72 induction was determined as follows. Cultures were simultaneously placed at 43°C. One culture was returned to 37°C after 1 h, one culture was returned to 37°C after 2 h, and the other culture remained at 43°C for the entire 3 h. Protein samples were harvested from all cultures 3 h after initiation of heat stress, and levels of HSP-72 expression were compared by Western blot analysis. HSP-72 expression was induced to high levels in TG cells regardless of heat stress duration (Fig. 8B). Therefore, HSV-1 reactivation in TG cells is not a direct result of triggering a heat shock response.

Analysis of potential reactivation-inducing drugs. It has been reported that LAT facilitates reactivation (16), and cAMP has been proposed to contribute to reactivation by upregulating LAT transcription (21, 29). Smith et al. (41) found that either 500 μ M CPT-cAMP or 50 μ M forskolin (i.e., an activator of adenyl cyclase) induced reactivation from neonatal neuron cultures. Likewise, epinephrine and glucocorticoids (e.g., Dex) have been used in animal models to induce reactivation of latent herpesvirus (19, 32, 46). Therefore, the reactivation-inducing potentials of cAMP, epinephrine, and Dex were studied in TG cell cultures to compare this system with other reactivation models.

While 0.5 μ M forskolin did not elevate cAMP above basal levels (1.4 ± 1.7 pmol of cAMP per mg of protein [mean \pm standard deviation]), cAMP levels were nearly 10-fold higher in TG cells 30 min after treatment with 50 μ M forskolin (12 ± 2.6 pmol of cAMP per mg of protein; mean \pm standard deviation). However, neither 50 μ M forskolin nor 500 μ M CPT-cAMP induced reactivation in TG cell cultures (Fig. 9). Epinephrine also had no effect in TG cell cultures. However, Dex induced reactivation in TG cells in a dose-dependent manner (Fig. 9). Following the 5-day period allowed for drug-induced reactivation, cultures were heat stressed to verify the presence of latent virus. Heat stress confirmed that in each treatment group, the majority of TG cell cultures contained reactivatable HSV-1 (i.e., 60 to 90% of cultures reactivated within 5 days after heat stress). As a control, some of the drug-treated cultures were not secondarily heat stressed; reactivation did not occur in these cultures.

Following heat stress, HSV-1 was detectable in culture supernatants of Dex-pretreated cells earlier than untreated controls. Likewise, comparison with results of previous experiments indicated that HSV-1 appeared significantly earlier in Dex-pretreated cultures (Table 2). Because of the known role of the glucocorticoid receptor as a transcriptional regulator (13, 31, 36), we postulated that Dex facilitated the induction of HSV-1 lytic-phase mRNA transcription following heat stress.

RT-PCR analysis of Dex- and vehicle-treated TG cells substantiated this hypothesis (Fig. 10). While viral mRNA was barely detectable in one of two vehicle-treated cultures 12 h after heat stress, viral transcripts for ICP27, thymidine kinase (TK), and ribonucleotide reductase (RR) were evident in two of two Dex-pretreated cultures 12 h after heat stress. Furthermore, detection of ICP27, TK, and RR mRNAs indicated that reactivation occurred in one of two DEX pretreated cultures that was not heat stressed. Because Dex was added just 15 h prior to extraction of RNA, the brief interval between stimulus and response suggests that Dex acted directly through neuronal glucocorticoid receptors to induce reactivation.

Despite the presence of ICP27, TK, and RR mRNAs, LAT was not detected in the Dex-treated culture that reactivated without being heat stressed. Interestingly, LAT was also not detected in vehicle-treated cultures 24 h after heat stress, despite the presence of lytic phase transcripts. On the basis of previous immunocytochemical staining experiments, RT-PCR was presumably detecting viral transcription in just one or two reactivation-positive cells. Therefore, the absence of LAT in these samples suggests a coordinated downregulation of LAT transcription during reactivation. Such an event has been proposed by Rock et al. (32) and could be mediated by ICP4, given the capacity of this HSV-1 regulatory protein to repress LAT transcription (1).

DISCUSSION

Establishment of TG cell cultures. The explantation of TG cells provides a reactivation-inducing stimulus to latently infected neurons during culture establishment. This is demonstrated by the upregulation of ICP27 mRNA expression in BVDU-treated and untreated cultures 24 to 48 h after culture establishment. Because the TG cells are plated as a single-cell suspension, however, the antiviral drug is effective immediately. Therefore, despite the induction of immediate-early gene expression, BVDU blocks HSV-1 DNA replication and prevents infectious virus production during culture establishment. The absence of ICP27 mRNA in BVDU-treated cells by 120 h after culture establishment indicates that the reactivation-inducing stimulus associated with explantation is tran-

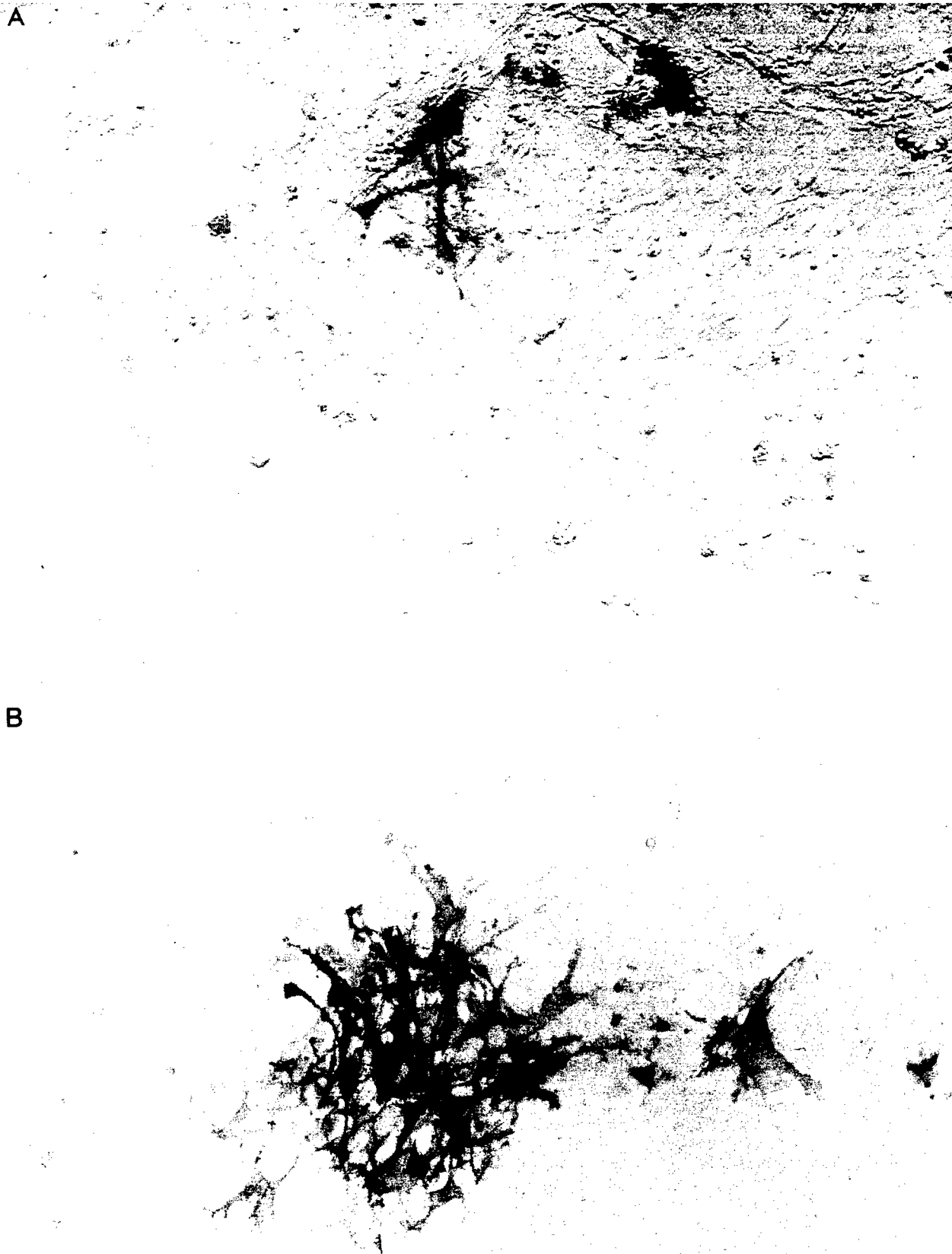


FIG. 6. Kinetics of HSV-1 protein induction following heat stress. TG cell cultures stained for HSV-1 antigen 22 h (A), 48 h (B), and 72 h (C) after initiation of heat stress (magnification, $\times 10$). (D) A neuron stained for HSV-1 antigen 22 h after heat stress (magnification, $\times 40$). All HSV-1 antigen-positive cells identified at this time point had a large nucleus and a prominent nucleolus, characteristic of neurons.

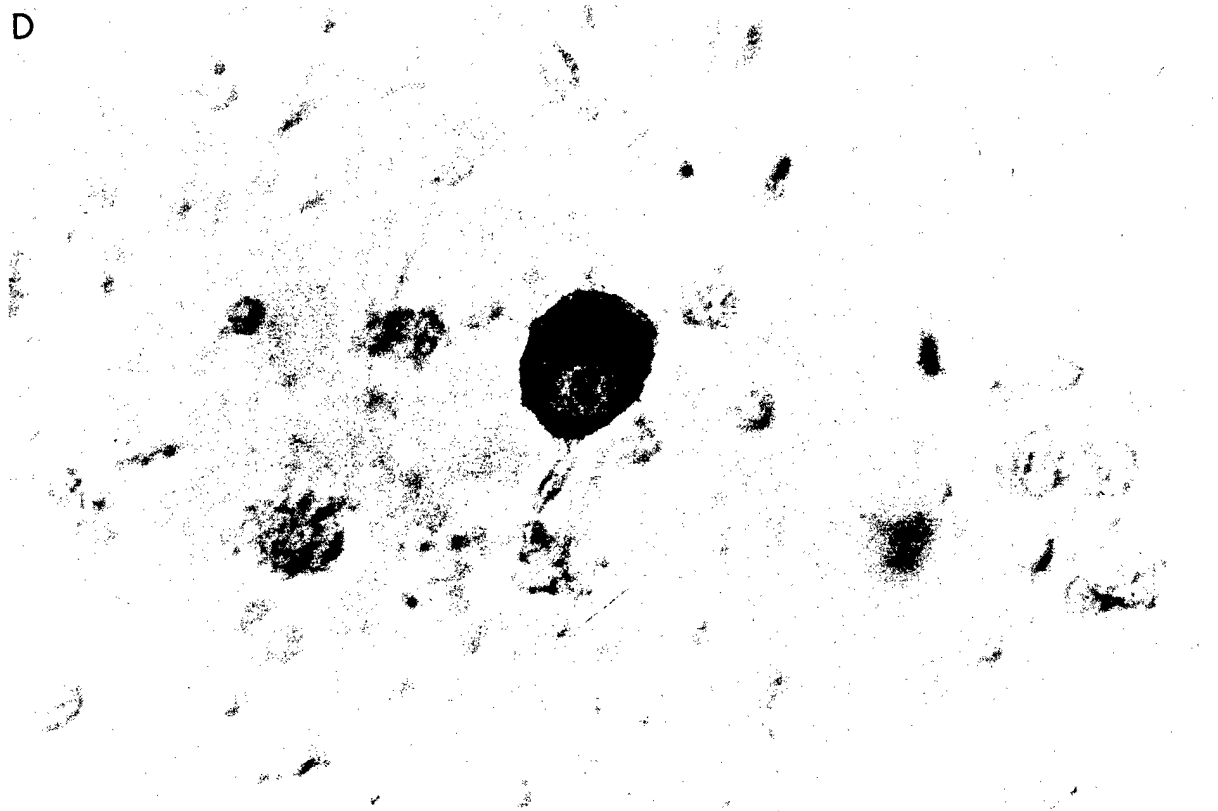


FIG. 6—Continued.

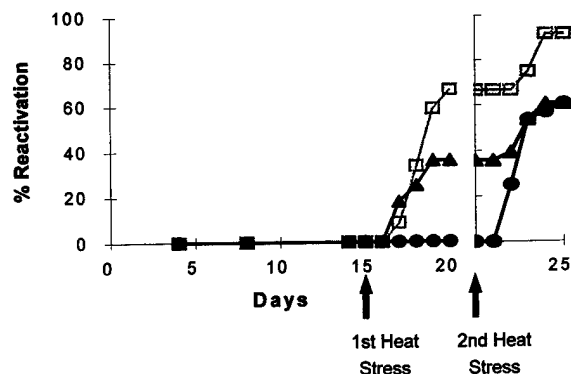


FIG. 7. Effect of heat stress duration on HSV-1 reactivation. Shown are percent reactivation following a 1-h (●), 2-h (▲), or 3-h (□) heat stress administered after 15 days in culture and percent reactivation following a second 3-h heat stress after 20 days in culture.

sient. Consistent with this hypothesis, throughout these studies reactivation was not observed following BVDU removal from TG cell cultures.

Reactivation from TG cells. In animal models, HSV-1 mRNA and antigen production in the ganglion is transient during stress-induced reactivation (2, 35). In light of evidence that T lymphocytes and cytokine expression persist in the TG after latency is established (4, 11, 40), the brevity of HSV-1 reactivation in vivo presumably reflects an efficient host immune response. In contrast, induction of reactivation in TG cell cultures is irreversible because lytic replication continues until all cells are destroyed.

Although HSV-1 remained largely undetected in culture medium until 48 h after heat stress, replication had initiated in neurons by 24 h after heat stress. The frequency of detecting HSV-1 in culture supernatants increased from 3 to 40% between 24 and 48 h after heat stress. HSV-1 antigen expression was restricted to neurons 22 h after heat stress but spread to plaque-like clusters of 50 to 500 cells by 48 h after heat stress. Likewise, RT-PCR indicated that the amount of ICP27 mRNA in TG cells increased substantially between 24 and 48 h after heat stress. Therefore, HSV-1 was not usually detected in culture medium until released from secondarily infected cells.

None of the treatments were 100% effective in inducing reactivation. In testing the effects of heat stress duration, 8 of 12 cultures reactivated after a first 3-h heat stress interval, but 3 of the remaining 4 cultures reactivated after a second 3-h heat stress. Likewise, while reactivation occurred in 16 of 28 cultures established in the absence of BVDU, 10 of the remaining 12 cultures reactivated following a secondary heat stress. Combined with the detection of LATs in 10 of 10 cultures, the results suggest that when cells are stressed, reactivation is initiated in only a fraction of latently infected neurons.

Mechanisms of HSV-1 reactivation. (i) Heat stress. While

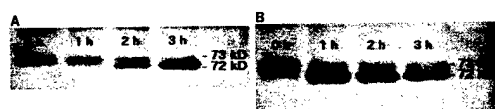


FIG. 8. Heat shock protein induction. (A) Kinetics of HSP-72 induction in TG cells 1, 2, and 3 h after transfer to a 43°C incubator (20 µg of protein per lane). (B) Effect of heat stress duration (1, 2, or 3 h) on heat shock protein induction by 3 h after transfer to 43°C incubator (35 µg of protein per lane).

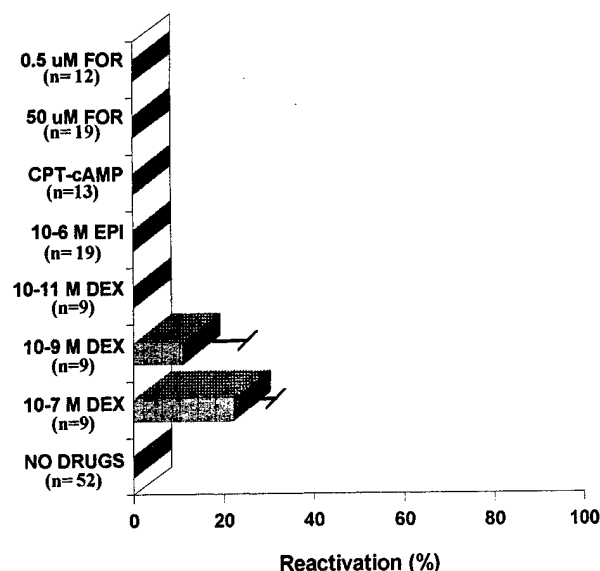


FIG. 9. Effects of drugs on reactivation. Frequency of drug-induced reactivation (\pm standard error of the mean) in TG cells treated with forskolin (FOR), CPT-cAMP, epinephrine (EPI), and Dex. Cultures were treated with drugs after 15 days in culture and observed for 120 h for reactivation.

HSP-72 expression was strongly induced in TG cells by 1 h of heat stress, reactivation occurred in 0 of 28 TG cell cultures incubated at 43°C for 1 h. Therefore, reactivation of HSV-1 gene expression is not a direct consequence of activating the signalling pathways which upregulate HSP-72 expression. However, heat stress induces HSP-72 expression more readily in glial cells than in neurons (34). Therefore, we cannot formally rule out the possibility that 1 h at 43°C induced HSP-72 expression in glial cells but was insufficient to activate the heat shock response in latently infected neurons.

(ii) Drugs. Stress stimulates epinephrine release from the sympathetic nervous system and glucocorticoid release from the adrenal glands. Given their roles as stress mediators, we hypothesized that these compounds serve as reactivation-inducing signals which act directly on latently infected neurons (i.e., ligand binding to neuronal adrenergic receptors or glucocorticoid receptors). In the case of epinephrine, this view is consistent with the finding that propranolol (β -adrenoceptor

TABLE 2. Enhancement by Dex of the rate of HSV-1 appearance following heat stress

Group	% reactivation ^a at indicated time poststress (mean \pm SEM)		
	24 h	48 h	72 h
10 ⁻⁷ M Dex ^b	26 \pm 11 ^c	50 \pm 17	100 \pm 0
10 ⁻⁹ M Dex ^b	0 \pm 0	90 \pm 10 ^c	100 \pm 0
10 ⁻¹¹ M Dex ^b	6 \pm 6	89 \pm 7 ^c	100 \pm 0
No Dex ^b	8 \pm 8	48 \pm 11	88 \pm 13
Expected ^d	2 \pm 4	46 \pm 13	84 \pm 6

^a Considering only those cultures that eventually reactivated, results represent percentages of reactivated wells at each time point after heat stress.

^b $n = 4$ experiments; four to five wells per experiment.

^c $P < 0.05$ (determined by analysis of variance and Tukey's post comparing Dex treatment groups with expected).

^d Pooled results of all other heat stress experiments ($n = 7$ experiments; 20 to 36 wells per experiment).

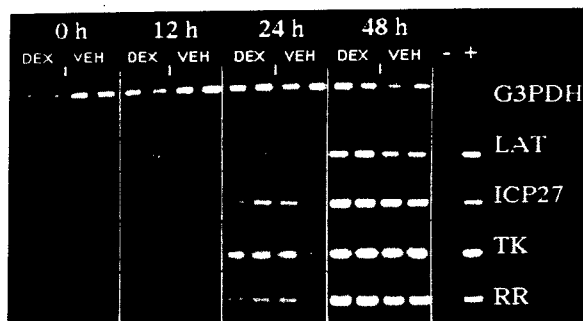


FIG. 10. Dex pretreatment enhances the rate of appearance of HSV-1 lytic-phase mRNA following heat stress. Shown is RT-PCR analysis of RNA from duplicate samples of TG cells prior to heat stress (0 h) and at 12, 24, and 48 h after initiation of heat stress. Dex, cultures which were treated with 10^{-7} M Dex 15 h before heat stress; VEH, cultures which were vehicle treated 15 h before heat stress. RT-PCR was used to detect G3PDH, LAT, ICP27, HSV-1 TK, and HSV-1 RR mRNAs. Each RNA sample was derived from the pooled cells of two culture wells. No template (-) and HSV-1 DNA (+) served as negative and positive controls, respectively, in each PCR assay.

antagonist) blocks reactivation from mice following hyperthermic stress (12).

Epinephrine did not induce reactivation of latent HSV-1 in TG cell cultures. Therefore, ocular iontophoresis of epinephrine may not act directly on neurons to induce HSV-1 reactivation in vivo. Rather, epinephrine iontophoresis may stimulate corneal cells, which in turn transmit a reactivation signal to the nerve endings of the ganglionic neurons. Denervation of the cornea has been shown to block epinephrine-induced reactivation of HSV-1 (33). Therefore, we cannot rule out the possibility that epinephrine fails to induce reactivation in vitro because the regenerated neurites of the neurons lack an essential component that is present in vivo. In contrast, because Dex and cAMP act on sites in the neuronal cell body, these results are more pertinent to the in vivo situation. Therefore, the inability of forskolin and CPT-cAMP to induce reactivation suggests that cAMP elevation is not a critical determinant of HSV-1 reactivation in ganglionic neurons.

Dex treatment induced HSV-1 reactivation in a fraction of TG cell cultures. Underwood and Weed (46) likewise found that glucocorticoids (i.e., prednisone) induced reactivation from a low but significant fraction (17%) of HSV-1 latently infected mice. Given these observations, one could hypothesize that while glucocorticoid receptors facilitate HSV-1 reactivation, other unidentified signals are necessary for efficient induction. Therefore, Dex may serve as more than an immunosuppressant when used in concert with cyclophosphamide and UV radiation to induce HSV-1 reactivation in animal models (7, 39). In contrast, Dex induces rapid reactivation of bovine herpesvirus 1 in nearly 100% of latently infected animals (8, 28, 32, 38, 47) and induces efficient reactivation of pseudorabies virus in latently infected swine (25).

There is clinical precedence for herpes simplex and herpes zoster reactivation following systemic corticosteroid treatment (3, 5, 17, 23, 27, 37). The results of the present study suggest that glucocorticoids may contribute to disseminated herpetic disease not only by suppressing immune function but also by providing a reactivation trigger to latently infected ganglion.

Application of the in vitro reactivation model. Because dissociated TG cell cultures mimic key aspects of in vivo latency and reactivation, this paradigm offers a valuable tool for studying aspects of reactivation that are not readily addressed in vivo. For example, antisense inhibition could be used in cultures to identify viral and cellular genes that modulate reactivation.

Likewise, reagents whose use is prohibitively expensive in vivo (e.g., recombinant proteins and monoclonal antibodies) can be used cost-effectively in vitro. Because the method used to detect reactivation does not require harvesting the cells, TG cell cultures are available for secondary manipulations after testing of potential reactivation-inducing stimuli. In contrast, detection of reactivation in vivo often requires sacrificing the host. Finally, TG cell cultures provide a means to identify treatments which act directly on ganglion cells to induce or block reactivation.

Inevitably with cell culture studies, one is left with the question, "How does this pertain to the in vivo situation?" At least with primary cell cultures, only the milieu is changed. Presumably in TG cultures, reactivation is induced from the same cells in which reactivation would occur in vivo. Therefore, we believe that this model can be used to identify molecular events associated with reactivation and generate hypotheses whose relevance can be determined in vivo. In this context, while the results of this study suggest a central role for glucocorticoid receptors in induction of HSV reactivation, additional in vivo studies are required to substantiate this hypothesis.

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Persistent Cytokine Expression in Trigeminal Ganglion
Latently Infected with Herpes Simplex Virus Type 1¹

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RUNNING TITLE: "CYTOKINE GENE EXPRESSION"

ABSTRACT

Following ocular infection, herpes simplex virus type-1 (HSV-1) establishes latency in trigeminal ganglion (TG) neurons. Using reverse transcription (RT)-PCR, cytokine gene expression was analyzed in the TG of HSV-1 infected mice. IL-2, TNF- α , IFN- γ , IL-10, and RANTES mRNA were readily detected in TG taken from mice 7 days post inoculation (PI). Likewise, IL-2, IL-6, IL-10, and IFN- γ protein were detected by ELISA of TG homogenates. Between 5-45 days PI, IL-10, IFN- γ , TNF- α , and RANTES mRNAs were detected in nearly 100% of latently infected TG, where latent infection was confirmed by RT-PCR detection of HSV-1 latency-associated transcripts (LAT). T cell-associated cytokine/chemokine mRNAs (IL-2, IL-10, IFN- γ , and RANTES) were still detected in the majority of latently infected TG taken between 60 and 135 days PI. In contrast, these cytokine mRNA species were rarely detected in uninfected TG. Measurement of serum Abs to HSV-1 at different times PI revealed that anti-HSV-1 Ab concentrations approached a plateau in mice by 30 days PI, but remained at high levels 67 and 125 days PI. While there was molecular evidence of an ongoing immune response to HSV-1 in latently infected TG, histologic analysis indicated that very few mononuclear cells remained in the ganglion 60 days PI. Collectively, the results suggest that residual lymphocytes encounter viral Ag during HSV-1 latency with sufficient frequency to remain activated. The paradox of a persistent immune response generated against a latent infection is discussed.

INTRODUCTION

Following primary infection of the eye or oral mucosa, herpes simplex virus type 1 (HSV-1)³ is carried from the epidermis to the trigeminal ganglion (TG) by retrograde transport along sensory nerve fibers (1). Viral Ag expression peaks in the TG 3 days post inoculation (PI) and rapidly induces an inflammatory response and elevated cytokine expression in the TG (2). By 10-14 days PI, HSV-1 replication ceases as a host immune response is mounted and latency is established. During latency, viral genomes are stably maintained in the neuronal nucleus as episomes which associate with cellular histones (3, 4). Viral transcription is generally thought to be limited to the expression of latency-associated transcripts (LAT) (5). Despite efforts to identify a functional open reading frame (6), a LAT protein has not yet been identified *in vivo*.

When viral replication spreads to the central nervous system (CNS) before an effective immune response is mounted, the outcome of primary HSV-1 infection is a fatal encephalitis. In susceptible strains, 5-10 days PI is the crucial period during which mice will either control the primary infection or become encephalitic. Differences in the susceptibility of mouse strains (i.e. C57BL/6 and BALB/c) to herpetic encephalitis has been attributed to a general decrease in permissiveness of C57BL/6 mouse cells for HSV-1 replication (7). However, there are also reports that immune factors such as MHC I haplotype (8) and IFN production (9) are critical determinants in controlling acute HSV-1 infection.

Regardless of the exact mechanism, it is important that an effective immune response is mounted before HSV-1 can spread unchecked into the CNS and cause encephalitis.

Five to ten percent of TG neurons become latently infected with HSV-1 following ocular infection (10, 11). Despite the extent of neuronal infection, less than 2% of ganglionic neurons are destroyed during acute HSV-1 infection (12). While there is some debate over the relative importance of CD4⁺ (13) and CD8⁺ (12) lymphocytes, T cells are generally considered to be essential for controlling HSV-1 replication and allowing the transition to latency (14-16). Because of the absence of extensive neuronal cell death, it appears that T lymphocytes shut down viral replication in the ganglion by non-cytolytic mechanisms. Specifically, T cell-secreted cytokines are believed to contribute to the efficient establishment of latency by inducing a nonpermissive state in ganglion cells (12, 17). Cytokines including IFN- α , IFN- γ , and TNF- α have been implicated in limiting HSV pathogenesis following primary infection (18,19).

If true latency of HSV-1 is established, cytokine expression in the TG should cease in the absence of viral Ag. However, several recent studies have shown evidence of a lingering cell-mediated immune response in the TG of latently infected mice. Immunohistochemical studies have detected IFN- γ -positive cells 6 months PI (20). Likewise, CD4⁺, CD8⁺, $\gamma\delta$ TCR⁺, and CD45RA⁺

lymphocytes are present in latently infected TG from 5 -92 days PI, along with significant numbers of macrophages (F4/80⁺ cells) (2, 20, 21). In the present study, evidence is provided that a cytokine response persists in latently infected TG for up to 4 months PI. Furthermore, serum anti-HSV-1 Ab concentrations in mice remain elevated 125 days PI. Collectively, the data raise the paradox of why immune effector cells persist at the site of a latent infection.

MATERIALS AND METHODS

Virus and Cells

The Vero and CV-1 African green monkey kidney cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI-1640 (Irvine Scientific, Santa Ana, CA) supplemented with 5% FBS (Gibco, Gaithersburg, MD) and an antibiotic / antimycotic solution (Atlanta Biologicals, Atlanta, GA). Cells were incubated at 37°C, 5% CO₂, and 95% humidity.

Mice were infected with HSV-1 (McKrae strain) which was grown as follows. Vero cells were infected with 0.1 - 0.5 plaque-forming units (pfu) HSV-1 per cell. After allowing 1 h at 37°C for viral adsorption, medium was discarded, cell monolayers were rinsed with PBS, and culture medium was added. Infected cells were incubated at 34°C until cytopathic effect was generalized (i.e. 48-72 h). Culture medium containing virus was centrifuged at 1000 x g for 10 min to remove cellular debris, and aliquots of the clarified supernatant were stored at -70°C.

Infection of Mice

Female ICR mice (25-34 g, Harlan-Sprague Dawley, Indianapolis, IN) were anaesthetized by intramuscular injection of ketamine (100 mg/kg) and intraperitoneal (i.p.) injection of xylazine (10 mg/kg). Following corneal scarification with a 25 g needle, tear film was blotted from the eyes with tissue, and mice were inoculated with HSV-1 by placing 3 µl of a 10⁵ pfu/ml solution (i.e. 300 pfu) on

each eye. Mice were given 0.1 ml rabbit antiserum to HSV-1 i.p. at the time of infection to enhance survival (22). Infection was verified by swabbing the eyes 2 days post-inoculation (PI), placing the swabs in CV-1 monolayer cultures and observing for cytopathic effects. Animals were handled in accordance with the National Institutes of Health guidelines on the Care and Use of Laboratory Animals, publication no. 85-23, revised 1985.

RT-PCR Analysis of Trigeminal Ganglia

To prevent contamination of TGs by cytokine-producing blood leukocytes, mice were sacrificed by CO₂ asphyxiation and bled by cutting the superior vena cava prior to opening the skull. Following dissection, TGs were rinsed in PBS and blotted on tissue paper to remove traces of blood. Left and right TGs were pooled and homogenized with a Pellet Pestle[®] Motor (Kontes, Vineland, NJ) in Ultraspec[®] RNA isolation reagent (Biotechx Inc., Houston, TX). RNA was extracted according to the manufacturer's instructions. First strand cDNA was synthesized from 4 µg total TG RNA using an oligo-dT₁₅ primer and AMV reverse transcriptase (Promega Corp., Madison, WI) in a 40 µl reaction volume. Because the major species of LAT is not polyadenylated (23), 20 ng of a LAT-specific oligonucleotide (ICP0-3') was used as a primer in a separate RT reaction containing 500 ng total TG RNA in a 10 µl reaction volume. Reverse transcription was performed according to the manufacturer's directions. TG cDNA (3.5 µl) was combined with 1X Taq buffer, 0.25 µM of each PCR primer, 100 µM of each dNTP, and 2.5 U of Taq

polymerase (Promega Corp.) in a 50 μ l reaction volume and overlaid with mineral oil. Oligonucleotide primers used in this study were synthesized by the LSU Medical Center Core Laboratories (New Orleans, LA) and are listed in Table I. PCR was performed in a MJ Research thermal cycler (Watertown, MA) with 35 cycles of 94°C (1'15") \rightarrow 57°C (1'15") \rightarrow 72°C (30"). PCR products were resolved in 2% agarose gels, and were visualized by ethidium bromide staining in an EagleEye II still video system (Stratagene, La Jolla, CA). Densitometric analysis of gel images was performed using ImageQuant 3.3 software (Molecular Dynamics, Sunnyvale, CA).

ELISA of cytokine proteins in TG

TGs from 5 mice were homogenized with a Pellet Pestle[®] Motor (Kontes) in 1.0 ml of carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) containing 50 μ g/ml bacitracin, 100 μ g/ml aprotinin, 100 μ g/ml pepstatin A, 100 μ g/ml benzamidine, and 5 μ g/ml phenylmethylsulfonylfluoride. Following centrifugation (1 min at 14,000 x g), IL-2, IL-6, IL-10, and IFN- γ concentrations in clarified homogenates were determined by ELISA (Pharmingen, San Diego, CA). The protein concentration in clarified TG homogenates was determined by the Bradford assay (BioRad Laboratories, Hercules, CA) according to the manufacturer's directions.

ELISA measurement of anti-HSV Ab titers

HSV-1 virion proteins were used as the coating Ag in ELISAs and were obtained as follows. Five 150 cm² flasks of CV-1 cells

were infected with 5 pfu HSV-1 per cell and incubated at 37°C for 24 h. HSV-1 infected cells were freeze-thawed 3 times in the culture medium. The cell lysate was clarified by centrifugation at 1000 x g for 10 min. Virions were pelleted onto a 60% sucrose cushion by centrifugation (20,000 rpm, 4°C, 4 h) in a Beckman 28 SW.1 rotor (Beckman Instruments, Irvine, CA). Virions were collected from the culture medium-sucrose interface using a syringe. The virion-containing fraction was diluted 1:5 in PBS, and the virions were then further purified over a discontinuous sucrose gradient (i.e. the virion fraction was applied to a gradient containing layers of 15%, 25%, 35%, and 60% sucrose, which was then centrifuged at 4°C in a Beckman 28 SW.1 rotor for 4 h at 20,000 rpm). Purified virions were collected from the interface between the 35 and 60% sucrose layers using a syringe, and the virion-containing solution was dialyzed against PBS.

ELISA to determine anti-HSV Ab titers was performed as follows. EIA 96-well plates (Costar, Cambridge, MA) were coated with 100 µl of HSV-1 virion proteins (diluted 1:50 in carbonate buffer) for 12 h at 4°C. Wells were blocked with 350 µl 0.5% dry milk dissolved in PBS for 1 h at 37°C. After rinsing once with PBS, duplicate 100 µl samples of diluted mouse serum (1:50 dilution in PBS) were added to HSV-1 Ag-coated wells and incubated for 1 h at 37°C. After rinsing 6 times with PBS (pH 7.4) containing 0.05% Tween-20® (polyoxyethylene-20-sorbitan monolaurate), hereafter referred to as T-PBS, 100 µl of affinity purified goat anti-mouse

IgG [H & L]-alkaline phosphatase conjugated Ab (Biorad Laboratories, Richmond, CA; diluted 1:1500 in PBS) was added to each well and incubated for 30 min at 37°C. After rinsing 6 times with T-PBS, 200 μ l of p-nitrophenyl phosphate (Sigma Chemical Co.) was added to each well and colorimetric development (OD₄₀₅) was measured in an ELISA plate reader (Bio-tek Instruments, Inc., Winooski, VT).

Statistics

Comparison of normally distributed data was performed by one-way ANOVA and Tukey's post hoc analysis. Comparison of non-parametric data was performed using Fisher's Exact Test. The correlation coefficient (of densitometric data) and its statistical significance were determined by regression analysis. In these statistical analyses, $p < .05$ was considered the minimum significant difference to reject $H_0: x_1 = x_2$.

RESULTS

Cytokine expression in TG during acute HSV-1 infection

Six to nine days after infection with the neurovirulent HSV-1 McKrae strain, one-third to one-half of infected ICR mice succumb to a fatal encephalitis. As viral replication spreads unchecked into critical areas of the brainstem and cerebellum, mice begin to display visible symptoms of encephalitis such as ataxia, hunched posture, and hypoexcitability (i.e. typically 24-36 h prior to death).

To determine if the presence or absence of certain cytokines correlated with encephalitis, cytokine expression was compared in the TG of mice that were or were not showing physical signs of encephalitis 7 days PI. ELISA of TG homogenates demonstrated that IL-2, IL-6, IL-10, and IFN- γ protein were present in acutely infected TG (Table II). However, IL-12 protein was not detectable by ELISA of TG homogenates. IL-10 and IFN- γ concentrations were significantly higher in TG of encephalitic mice relative to non-encephalitic mice 7 days PI (Table II). However, these differences were not evident at the mRNA level by RT-PCR analysis (Figure 1.a). While there was no consistent correlation between HSV-1 lytic phase replication in the TG and encephalitis (Figure 1.a), encephalitis correlated well with HSV-1 replication in the cerebellum. ICP27 transcripts were abundant in the cerebellum of encephalitic mice, but were not detected in the cerebellum of non-encephalitic mice 7 days PI (Figure 1.b).

Densitometric analysis of ethidium bromide-stained RT-PCR products indicated that a strong correlation existed between the yield of ICP27 and cytokine (i.e. RANTES and TNF- α) RT-PCR products amplified from TG 5-7 days PI (Figure 2). The parallel increase in ICP27 and cytokine RT-PCR product yields was not due to differences in the quality of TG cDNA samples, because G3PDH RT-PCR product yield from these samples was very similar (i.e. 20300 ± 650 pixels, mean \pm sem). The correlation coefficient between RT-PCR product yields of ICP27 and RANTES was 0.94 ($p = .0002$), and between ICP27 and TNF- α was 0.89 ($p = .001$). Presumably, increased amounts of the cytokine mRNAs reflect a greater inflammatory infiltrate in the TG in response to larger virus loads.

Cytokine gene expression in TG during HSV-1 latency

Using RT-PCR, cytokine transcription was studied in the TG during the transition from acute to latent HSV-1 infection. RT-PCR detection of LAT RNA was used to confirm that TG were infected with HSV-1 (Figure 3). Because ICP27 is essential for productive HSV-1 infection, the absence of ICP27 mRNA confirmed that TG were latently infected after 14 days PI (Figure 3). While cytokine mRNA was not found in uninfected TG, cytokine transcript expression (especially IL-10 and IFN- γ mRNA) was strongly upregulated in acutely infected TG 5 days PI (Figure 3). IL-2, IL-10, IFN- γ , RANTES, and TNF- α mRNA persisted in TG well after active HSV-1 replication had ceased; cytokine transcripts were detected in TG 14, 24, 35, 60, and 125 days PI (Figure 3).

In total, cytokine expression was evaluated in 27 uninfected mice and 91 HSV-1 infected mice sacrificed between 5 and 135 days PI (Table III). The presence of LATs was confirmed in $84 \pm 5\%$ of infected TG samples by RT-PCR (hereafter referred to as LAT⁺ TG). When appropriate measures were taken to avoid contamination of TG with blood during dissection (see Materials & Methods), cytokine mRNAs were rarely detected in TG from uninfected mice (Table III). In contrast, IL-10, IFN- γ , RANTES, and TNF- α mRNA were detected in nearly 100% of LAT⁺ TG between 5 and 45 days PI (Table III). The frequency of detecting IL-10, IFN- γ , and RANTES mRNA in LAT⁺ TG decreased significantly by 110-135 days PI, but there was still over a 50% correlation between latent HSV-1 infection and the presence of cytokine transcripts (Table III). Expression of IL-10, IFN- γ , RANTES, and TNF- α mRNA was highly correlated. Typically, either all four cytokine mRNAs were detected in LAT⁺ TG, or none of the cytokines were detected. The correlation between presence of IL-2 mRNA and the other cytokine mRNAs was more variable.

Comparison of LAT⁺ and LAT⁻ TG

Despite the fact that primary infection was verified in mice by ocular swabbing 2 days PI, LAT RNA was not detected in 16% of TG samples from infected mice. There was a strong correlation between detection of LAT and the presence of cytokine mRNA in TG (Figure 4). Because there was no difference in the amount of G3PDH PCR product amplified from LAT⁺ and LAT⁻ TG (as determined by densitometric analysis), the failure to detect LAT and cytokine

mRNA in LAT⁻ TG was not simply due to differences in the quality of cDNA samples.

IFN- γ , RANTES, and TNF- α mRNA were detected significantly less frequently in LAT⁻ TG 5-14 days PI relative to LAT⁺ TG taken 5-14 days PI (Table V). While IFN- γ , RANTES, and TNF- α mRNA were detected in 75-80% of LAT⁺ TG taken 30-135 days PI, IFN- γ and RANTES mRNA were detected in none of the LAT⁻ TG taken 30-135 days PI. Likewise, TNF- α mRNA was detected in only 25% of LAT⁻ TG (Table V). In contrast to these other cytokines, IL-10 was detected in 4 of 5 LAT⁻ TG taken 7-14 days PI and in 3 of 8 LAT⁻ TG taken 30-135 days PI.

ELISA detection of anti-HSV-1 Abs in serum from mice correlated with RT-PCR detection of LAT in TG. While anti-HSV-1 Abs were found in serum from mice whose TG were LAT⁺ by RT-PCR, serum anti-HSV-1 Abs were not detected in mice whose TG were LAT⁻ by RT-PCR (Table IV). Given this evidence that RT-PCR detection of LAT in TG was an accurate indicator of latent HSV-1 infection, the difference in cytokine mRNA profiles between LAT⁺ and LAT⁻ TG was not unexpected.

Anti-HSV-1 Ab titers do not decrease during HSV-1 latency

After the acute infection, serum concentrations of anti-HSV-1 Abs increased steadily in mice from 7-30 days PI, and reached a plateau by 50 days PI (Figure 5). In the absence of viral Ag

during latency, serum anti-HSV-1 Ab titers should peak after the resolution of acute infection and gradually decline. In fact, however, serum Abs against HSV-1 virion proteins were still present in high concentrations in mice 67 and 125 days PI (Figure 5). The relative anti-HSV-1 Ab concentration in serum samples was derived by fitting sample ODs to a standard curve based on a two-fold dilution series of high titer anti-HSV-1 mouse serum ($r^2=.995$). Based on the calculation, serum concentrations of anti-HSV-1 Abs were 1.5, 3.1, and 4.9 fold higher on average at 21, 50, and 125 days PI, respectively, relative to serum taken 14 days PI.

Histology of latently infected TG

The histology of TG sections from mice sacrificed 14, 30, 62, 108, 121, and 135 days PI was compared to that of uninfected TG. Increased numbers of IFN- γ^+ , IL-10 $^+$, and TNF- α^+ cells were evident by immunohistochemical staining of HSV-1 infected TG taken between 14-30 days PI, relative to uninfected control sections (data not shown). However, few differences were noted between uninfected TG and HSV-1 infected TG taken 62, 108, 121, and 135 days PI.

Relative to uninfected TG (Figure 6.A), an intense monocyte infiltrate was evident in TG 14 days PI during the resolution of acute HSV-1 infection (Figure 6.B). Likewise, areas of mononuclear cell infiltration were apparent among neuronal cell bodies in TG 30 days PI (data not shown). However, by 62 days PI histological evidence of an ongoing immune response was difficult to find. Of

16 TG examined between 62 and 135 days PI (i.e. 4 TG per time point), only one neuron in a TG taken 108 days PI appeared to be the target of a mononuclear cell infiltrate (Figure 6.C). The focal nature of the infiltrate suggests an immune response to a neuron producing viral Ags. Although this a speculative point, such an infrequent, localized event may account for the persistence of cytokine-expressing cells in latently infected TG (4 months PI), suggesting that a small population of lymphocytes is stimulated by viral Ag during latency.

DISCUSSION

Cytokine expression during acute HSV-1 infection

The correlation between the presence of HSV-1 transcripts (i.e. ICP27 or LAT) and cytokine transcripts (i.e. IL-10, IFN- γ , RANTES, and TNF- α) in the TG was nearly 100% during resolution of the acute infection. The observation that T cell-depleted mice do not survive primary HSV-1 infection (24) demonstrates the importance of these effector cells and their secreted cytokines in controlling the primary infection. Cytokines including IFN- α , IFN- γ , and TNF- α may also act directly on ganglion cells to block HSV-1 replication by inducing a non-permissive state (25-27). This type of cytokine-mediated control of viral replication has been demonstrated in hepatitis B virus (HBV) transgenic mice. IFN- γ and TNF- α produced by HBV-specific CTLs block viral gene expression by inducing the selective degradation of HBV mRNA in hepatocytes (28-30).

Cytokine expression in latently infected TG

The present study demonstrates that TNF- α and T cell-associated cytokine transcripts (IL-10, IFN- γ , and RANTES) are consistently present in latently infected TG 1-2 months PI. Cantin, et al. (1995) observed large numbers of IFN- γ ⁺ cells 180 days PI in latently infected TG by immunohistochemical staining (20). In contrast, Liu, et al. (1996) observed only a few IFN- γ ⁺, IL-4⁺, and IL-10⁺ cells in latently infected TG taken 30 and 92 days PI, but found many TNF- α ⁺ cells at these later time points (2).

Our results are more consistent with the latter study, indicating that T cell cytokine expression persists well into latency, but only occurs at low levels after 30-45 days PI.

The observation that T lymphocytes persist in latently infected TG has now been reported in A/J, BALB/c, and NIH/OLA mice infected with HSV-1 strains RE, F, and McKrae, respectively (2, 20, 21). In the present study, outbred ICR mice were infected with HSV-1 (McKrae). Therefore, the persistence of T cells and cytokine expression in HSV-1 latently infected mouse ganglion is not a strain-specific phenomenon. However, this event may be unique to mice because T cells are not detected in rabbit TG 30 days PI by immunohistochemical staining (31).

Based on the use of immunosuppressive treatments (i.e. X-ray irradiation, cyclophosphamide) to induce reactivation from latently infected mice (32), one could argue that cytokine expression is necessary for the maintenance of HSV-1 latency in the ganglion. However, because of the systemic stress induced by these treatments, one cannot be sure that reactivation strictly follows from the immunosuppression. Furthermore, in an *in vitro* HSV-1 reactivation model, spontaneous reactivation does not occur from latently infected TG neurons (33) despite the absence of IFN- γ and little to no TNF- α in culture supernatants⁴. Therefore, while cytokine expression appears to be important in controlling primary HSV-1 infection (20), the role of cytokines during latency remains

unclear.

Implications regarding the nature of HSV-1 latency

The nature of HSV-1 latency has long been debated. It has been suggested that there may be a "trickle effect" such that low level HSV-1 replication occurs in latently infected TG. However, infectious virus is only infrequently recovered from tear film and TG of latently infected mice (34,35). Therefore, HSV-1 is believed to establish a truly latent infection in mice where spontaneous reactivation rarely occurs.

Low level expression of HSV-1 proteins during latency could account for lymphocyte infiltration and T cell activation (i.e. cytokine expression) in latently infected TG. Immunoreactive ICP4 has been identified in latently infected rabbit TG (36), and more recently, RT-PCR has been used to demonstrate low level ICP4 and HSV-1 thymidine kinase mRNA expression in latently infected mouse TGs (37). Likewise, LATs may encode an as of yet unidentified protein *in vivo*, such as the latency-associated antigen identified *in vitro* (38). However, latent HSV-1 has only been detected in neurons (10, 39), a cell type which does not express MHC I (40) and is therefore incapable of presenting endogenous Ags to CD8⁺ T cells. Therefore, to accept the hypothesis that persistent cytokine expression in TG results from low level viral Ag expression in latently infected neurons, one has to believe that either low level MHC I expression occurs in neurons, or that other

antigen presentation pathways exist.

Other possible sources of Ag that could account for the persistence of T cells in latently infected TG are 1) self Ags exposed during HSV-1 infection (41), 2) long-lived viral Ag which persists after the acute infection, 3) low level production of secreted viral Ags which are processed and presented by APCs (e.g. macrophage) to T lymphocytes, and 4) reactivation of infectious virus from latently infected neurons. While HSV-1 reactivation seems a plausible source of Ag to account for T cell persistence in latently infected ganglion, infectious virus is only rarely detected in mouse TG after the acute infection (33, 34). Perhaps the explanation for this discrepancy is that periodic sampling for infectious virus is less sensitive than host immune cells which are continuously present. In the natural host (i.e. humans), HSV-1 efficiently reactivates in the face of humoral and cell-mediated immunity (42). Perhaps HSV-1 is unable to evade immune recognition during reactivation in mice, and so only small quantities of infectious virus are produced.

Regardless of the specific mechanisms, the data presented herein suggest that viral Ag is present in sufficient quantities during latency to stimulate an ongoing immune response. Further investigation is necessary to establish the underlying cause of this phenomenon.

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FOOTNOTES

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3. Abbreviations used in this paper: HSV-1, herpes simplex virus type 1; TG, trigeminal ganglion; PI, post inoculation; LAT, latency-associated transcript; ICP27, infected cell polypeptide 27.

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FIGURE LEGENDS

Figure 1. Cytokine transcription in acutely infected TG. (A) RT-PCR analysis of triplicate samples of TG from uninfected mice (UI), and either non-encephalitic (-) or encephalitic (+) mice sacrificed 7 days PI. From top to bottom, RT-PCR was used to detect the following mRNAs in TG samples: the housekeeping gene glyceraldehyde-3-phosphodehydrogenase (G3PDH), HSV-1 latency-associated transcripts (LAT), HSV-1 infected cell polypeptide 27 (ICP27), IL-2, IL-10, IFN- γ , RANTES, and TNF- α . (B) RT-PCR of cerebellum samples from the same 3 uninfected (UI), non-encephalitic (-), and encephalitic (+) mice shown in Figure 1.A analyzed for the presence of G3PDH and HSV-1 ICP27 mRNA.

Figure 2. Correlation between cytokine and viral RT-PCR product yield amplified from HSV-1 infected TGs. Each point represents the cytokine RT-PCR product yield versus ICP27 RT-PCR product yield amplified from a TG during the acute infection. Each pixel in the gel image is assigned a value between 0 and 255. RT-PCR product yield is therefore expressed in terms of the integrated volume of pixels associated with each ethidium bromide-stained band on an agarose gel.

Figure 3. Cytokine transcription in TG following HSV-1 infection. RT-PCR analysis of duplicate samples of TG taken from uninfected (UI) mice, and from mice sacrificed 5, 14, 24, 35, 60, and 125 days post-inoculation (PI). This figure is a representative example of

RT-PCR experiments summarized in Table 3. From top to bottom, RT-PCR was used to detect the following mRNAs in TG samples: the housekeeping gene G3PDH, HSV-1 LAT, HSV-1 ICP27, IL-2, IL-10, IFN- γ , RANTES, and TNF- α .

Figure 4. Comparison of cytokine mRNA profiles in LAT⁺ and LAT⁻ TG. RT-PCR analysis of representative TG samples taken from mice 37-42 days PI. From top to bottom, RT-PCR was used to detect the following mRNAs: the housekeeping gene G3PDH, HSV-1 LAT, IFN- γ , RANTES, and TNF- α .

Figure 5. Anti-HSV-1 Ab concentrations at times after infection. ELISA measurement of anti-HSV-1 Abs (OD₄₀₅) in mouse serum is shown versus time after inoculation (Days PI). Results represent mean \pm sem OD₄₀₅ and are based on serum obtained from mice 7 days (n=4), 14 days (n=4), 21 days (n=20), 33 days (n=10), 50 days (n=5), 70 days (n=6), and 125 days (n=9) PI.

Figure 6. Mononuclear cell infiltration in TG. Hemotoxylin-eosin-stained 8 μ m sections (20x magnification) of an a) uninfected TG, b) HSV-1 infected TG (14 days PI), and c) HSV-1 latently infected TG (108 days PI). Arrows point to areas of mononuclear cell infiltration. Histologic analysis was performed on 4 TG at each time point (14, 30, 62, 108, 121, 135 days PI). To obtain a representative view of the TG, at least 12 sections (4 adjacent sections from 3 different planes within the TG) were analyzed.

TABLE I. Oligonucleotide primers.

Primer	Product Size (bp)	Sequence
G3PDH-a	239	GAATCTACTGGCGTCTTCACC
G3PDH-b		GTCATGAGCCCTTCCACGATGC
ICP0-3'	-	TTCGACCAGGGCACCCTAGT
LAT-a	195	GACAGCAAAAATCCCCTGAG
LAT-b		ACGAGGGAAAACAATAAGGG
ICP27-a	283	TTTCTCCAGTGCTAGCTGAAGG
ICP27-b		TCAACTCGCAGACACGACTCG
IL-2-a	247	TCCACTTCAAGCTCTACAG
IL-2-b		GAGTCAAATCCAGAACATGCC
IL-10-a	256	GGACAACATACTGCTAACCGAC
IL-10-b		AAAATCACTCTTCACCTGCTCC
IFN- γ -a	237	AACGCTACACACTGCATCTTGG
IFN- γ -b		GACTTCAAAGAGTCTGAGG
RANTES-a	271	GAAGATCTCTGCAGCTGCCCT
RANTES-b		GCTCATCTCCAAATAGTTGA
TNF- α -a	384	GCCTGTAGCCACGTCGTAG
TNF- α -b		TTGGGCAGATTGACCTCAGC

TABLE II. Cytokine concentrations^a in TG homogenates.

[Cytokine]	Encephalitic	
	-	+
IL-2 (pg/ml)	331 ± 72	466 ± 59
IL-6 (pg/ml)	1021 ± 281	1201 ± 258
IL-10 (pg/ml)	126 ± 8	213 ± 21 ^{^^}
IFN-γ (U/ml)	ND ^b	17.5 ± 3.9 [^]

^a Results represent mean ± sem cytokine concentration (based on 3-5 independent experiments), as determined by ELISA of supernatants from the pooled homogenates of 5 pairs of TGs.

^b Not detectable (i.e. < 10 U/ml).

[^] p < 0.05

^{^^} p < 0.01

TABLE III. Cytokine transcription in LAT⁺ TG.

PI Day	Percent cytokine-positive TG ^a				
	IL-2	IL-10	IFN- γ	RANTES	TNF- α
UI ^b (n=27)	8 \pm 6	0 \pm 0	6 \pm 6	0 \pm 0	3 \pm 3
5-7 ^c (n=12)	63 \pm 19	100 \pm 0	100 \pm 0	100 \pm 0	93 \pm 7
14-24 ^d (n=7)	83 \pm 17	92 \pm 8	100 \pm 0	100 \pm 0	100 \pm 0
30-45 ^e (n=11-34)	68 \pm 22	84 \pm 10	91 \pm 5	94 \pm 4	93 \pm 7
60-67 ^f (n=5-8)	80 ^g	57 \pm 23 [*]	80 \pm 10	70 \pm 30	80 \pm 10
110-135 ^h (n=10-15)	38 \pm 5	63 \pm 3 [*]	56 \pm 21 [*]	52 \pm 21 [*]	67 \pm 24

^a Results represent mean \pm sem percent detection of cytokine transcripts in LAT⁺ TG, as determined by RT-PCR.

^b Pooled results of 9 experiments.

^c Pooled results of 3 experiments.

^d Pooled results of 3 experiments.

^e Pooled results of 7 experiments.

^f Pooled results of 2 experiments.

^g Result of a single experiment.

^h Pooled results of 4 experiments.

^{*} $p < .05$, determined by ANOVA & Tukey's post comparing latent to acute TG (5-7 days PI).

TABLE IV: ELISA of serum anti-HSV-1 Abs.

Days PI	Anti-HSV-1 Ab ^a	
	LAT ⁻	LAT ⁺
0 ^b	0.031 ± 0.009	---
35 ^c	0.022 ± 0.013	0.277 ± 0.068 [*]
125 ^d	0.012 ± 0.013	0.614 ± 0.022 ^{***}

^a Results represent mean ± sem OD₄₀₅ of anti-HSV-1 Ab ELISA on mouse serum. 'LAT+' = LAT RNA was detected in mouse TG. 'LAT-' = LAT RNA was not detected in mouse TG by RT-PCR.

^b n=3; 0 days PI indicates uninfected mice.

^c n=6 per group.

^d n=3 per group.

^{*} p < .01, determined by ANOVA & Tukey's post comparing sample groups to OD₄₀₅ of serum from uninfected mice.

^{***} p < .0001, determined as above.

TABLE V. Cytokine mRNA detection in LAT⁺ and LAT⁻ TG.

Days PI	LAT ^b	Fraction of cytokine-positive TGs ^a				
		IL-2	IL-10	IFN- γ	RANTES	TNF- α
7-14	+	8/12	12/12	12/12	12/12	12/12
	-	1/5	4/5	2/5 [*]	1/5 ^{**}	1/5 ^{**}
30-135	+	13/26	40/53	30/40	43/57	28/34
	-	1/4	3/8	0/8 ^{***}	0/10 ^{***}	2/8 ^{**}

^a Results are expressed as [cytokine⁺ TG / total number of TG].

^b Indicates the presence (+) or absence (-) of HSV-1 latency-associated transcripts, as determined by RT-PCR.

^{*} $p < .05$, determined by Fisher's Exact Test comparing LAT⁺ and LAT⁻ TG within either the 7-14 days PI group or 30-135 days PI group.

^{**} $p < .01$, determined as above.

^{***} $p < .001$, determined as above.

754

ANALYSIS OF THE IMMUNE RESPONSE DURING STRESS-INDUCED REACTIVATION OF HERPES SIMPLEX VIRUS TYPE 1 FROM LATENCY. William P. Halford, Bryan M. Gahard, and Daniel J.J. Carr. Departments of Micro. & Immunology, Pharmacology, Ophthalmology, LSU Medical Center, New Orleans, LA, USA.

Reactivation of herpes simplex virus type-1 (HSV-1) from latency occurs in response to stress. The stress response clearly provides a stimulus that reactivates viral gene expression. However, stress mediators (e.g. glucocorticoids) may contribute secondarily to HSV-1 transmission by enhancing the survival of infectious virus shed at the source. Using a hyperthermic stress paradigm, we are studying changes in endocrine stress mediators and the local immune response during viral reactivation. 24 hours after exposure to this stressor, latently-infected mice have significantly elevated serum corticosterone levels relative to both uninfected, stressed and infected, non-stressed controls ($p < .05$). Likewise, while serum concentrations of corticosterone and IL-6 are positively correlated in uninfected, stressed mice ($r = .87$), no such correlation exists in infected, stressed mice ($r = -.28$). Taken together, these results suggest that HSV-1 is capable of modifying the stress response of its host, presumably in a way that favors viral reactivation and horizontal transmission. With regard to the local immune response, RT-PCR analysis of trigeminal ganglia RNA indicate that the cytokines TNF- α and IL-1 play an early role in the neural/immune response to stress-induced HSV-1 reactivation.

This work was supported in part by grants from the U.S. Public Health Service, NIH/NEI EY08071, and the Department of the Army, Cooperative Agreement DAMD17-93-V-3013.

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ANTI-HCMV ACTIVITY OF CYTOKINES PRODUCED BY IE1-SPECIFIC CD4⁺ T CELL CLONES IN VITRO. P. Castan  , J. Allan Yorke, C. Davrinche, and J.-L. Davignon. INSERM U395, BP 3028, 31024 Toulouse, France.

We have previously shown that the precursor frequencies of IE1-specific proliferative T cells are high in healthy HCMV seropositive blood donors. In order to study the role of anti-IE1 CD4⁺ T cells in anti-HCMV immune response, we have tested TH1-type cytokines (IFN- γ , TNF- α), either recombinant or produced by T cell clones, on *in vitro* infection by HCMV (Towne). Flow cytometry experiments showed that preincubation of U373 MG cells with recombinant IFN- γ and TNF- α markedly diminished the expression of proteins detected by E13 and CCH2 mAbs on day 5 post infection (p.i.). These cytokines had a synergistic effect. However, on day 2, their combination induced only a slight inhibition of IE expression. Similarly, quantitative RT-PCR showed no reduction of IE1 mRNA by rIFN- γ 24h p.i. Plaque reduction assay on MRC5 cells showed synergistic effect of rIFN- γ and rTNF- α on virus replication. Anti-IE1 CD4⁺ clones produced IL-2, large amounts of IFN- γ and TNF- α , but no IL-6, when cultured in the presence of Antigen Presenting Cells (APC) and specific IE1 peptide. Preincubation of U373 MG cells with supernatant of specifically activated anti-IE1 CD4⁺ clone reduced the expression of the protein detected by CCH2 mAb to almost undetectable level on day 5 p.i. Coculture of CD4⁺ clone and APC plus IE1 peptide with U373 MG cells, previous to HCMV infection, drastically reduced virus production. The anti-HCMV activity of CD4⁺ clone supernatant could be inhibited using anti-IFN- γ + anti-TNF- α antibodies. This indicated that the anti-HCMV activity contained in CD4⁺ clone supernatant was due to IFN- γ and TNF- α . Our results, which show that activated anti-IE1 CD4⁺ clones display anti-HCMV activity *in vitro*, suggest a possible role for anti-IE1 CD4⁺ T cells in the anti-HCMV response.

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ANTIBODY RESPONSE OF PORPHYROMONAS GINGIVALIS INFECTED GINGIVITIS AND PERIODONTITIS SUBJECTS. E. Gemmell, B. Polak, RA Reinhardt, G.J. Seymour. Oral Biology and Pathology, Department of Dentistry, The University of Queensland 4072 Australia. Department of Periodontology University of Nebraska Medical Center College of Dentistry Lincoln NE

Porphyromonas gingivalis demonstrates a strong association with adult periodontitis although some individuals with the infection are able to control it without experiencing attachment loss. Therefore differences in the immune response to this organism may be of importance to the outcome of periodontal disease. The aim of this study was to determine whether *P. gingivalis* positive subjects with and without periodontal breakdown, reacted differently to *P. gingivalis* antigens as assessed by serum antibody reactivity. Two highly defined groups of subjects were chosen for this study. Both demonstrated *P. gingivalis* in their plaque and both had responded to *P. gingivalis* as shown by the presence of serum antibodies. The two groups differed only in their clinical susceptibility (adult periodontitis) or resistance (gingivitis) to periodontal breakdown. Western blots of *P. gingivalis* outer membrane (OM) antigens were probed with sera from subjects in the two groups to determine their reactivity to specific antigens. Analysis of the immunoblots showed that there were no differences in either the total numbers of bands, or bands recognized by the majority of subjects in the gingivitis and adult periodontitis groups. There were however, 4 bands which were recognized by the majority of the gingivitis group and not by the majority of the adult periodontitis group. Of these, there was a significant difference ($p < 0.01$) in the recognition of a 91.4 kDa antigen band. A further 5 antigens of lower molecular weight were seen by the majority of the adult periodontitis group and not by the majority of the gingivitis group. When sera were tested against purified *P. gingivalis* LPS, the results indicated that the 5 antigens seen by the majority of the adult periodontitis group may be LPS antigens. These results suggest that subjects susceptible and non-susceptible to periodontal disease may recognize different *P. gingivalis* antigens.

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CAN A STRIP METHOD (IMMUNODOT) CONTRIBUTE TO THE IMMUNODIAGNOSIS OF LYME BORRELIOSIS? J. Strakos  , R. Martinka, B. Moric, M. Vrljik, J. C  p, D. Sonora, P. Bakovick  . Department of Clinical Immunology and Internal Medicine I, Martin Faculty Hospital and Jessenius Medical School, Martin, Slovakia.

In the efforts focused on selecting a simple, quick and inexpensive immunodiagnostic method of Lyme borreliosis, a strip method (IMMUNODOT Borrelia (Lyme) with recombinant Antigen P39, Epignot (Linz, Austria) was tested against the Indirect fluorescent antibody method (IFAB), Sevaest Borrelia IgG set IFA, Sevac, USOL (Prague, Czech Republic). In 87 patients exhibiting clinical signs suspect for Lyme disease the results of both methods were correspondingly positive in 43 (49.4%) patients which was considered as serologic confirmation of the diagnosis and antibiotic treatment was introduced. Negative results of both tests were in 21 (24.1%), and different results in 23 (26.4%) patients. Of the latter in 14 (16.1%) Immunodot was slightly positive (1:64, 1:128) while IFAB was negative (1:16, 1:32) and in 9 (10.3%) patients with negative Immunodot the results of IFAB were slightly positive (1:64, 1:128). In clinically positive patients with inconsistent results of immunotests (IFAB negat. - Immunodot posit., IFAB slightly posit. - Immunodot negat.) we recommended to introduce the ATB treatment and to re-test them after 1-2 months. Since the Western Blot as a confirmation test and the new tests with recombinant antigens are both very demanding and expensive techniques in our conditions, we can consider Immunodot a positive contribution to the specific diagnosis of the Lyme disease. However, clinical diagnosis at the time being, is the most important for therapeutic considerations.

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T CELL EPITOPE ANALYSIS IN THEILER'S VIRUS - INDUCED DEMYELINATING DISEASE. R.L. Yauch, K. Sawani and B.S. Kim, Departments of Pathology and Microbiology-Immunology, Northwestern University Medical School, Chicago, IL, USA

The intracerebral inoculation of Theiler's Murine Encephalomyelitis Virus (TMEV) into susceptible strains of mice results in a chronic, immune-mediated demyelinating disease that shares many features with human Multiple Sclerosis. CD4⁺ T lymphocytes are critically important for pathogenesis, therefore determining the fine epitope specificity of the T cell response is essential for elucidating the mechanism(s) involved in demyelination. We have produced a panel of T cell hybridoma clones from the spleens of TMEV-infected, SJL/J mice and have analyzed the fine epitope specificity of these T cells. Although many of the T cell hybrids were specific for previously identified epitopes within VP1₂₃₅₋₂₅₀ (6/17) and VP2₁₋₄₆ (3/17), there were several TMEV-specific hybridomas with an unknown fine epitope specificity. Through the use of fusion proteins and synthetic peptides, we have determined that these hybridomas are specific for the amino acids 24-37 of the VP3 capsid protein. Strong T cell proliferative responses are detected against VP3₂₄₋₃₇ from both virus-immunized and virus-infected, SJL/J mice, and this response is I-A^b and CD4⁺ restricted. Precursor frequency analyses, as well as an analysis of virus-specific T cell hybridomas, suggest that VP3₂₄₋₃₇ - specific T cells also represent a predominant population of virus-specific T cells. A comparison of the T cell response to the major epitopes of TMEV demonstrated that there is no preferential response to a specific epitope at any time post-infection that was analyzed. Furthermore, immunization with epitope-containing peptides, following an injection with a suboptimal dose of live virus, results in the exacerbation of demyelinating disease. These results would strongly suggest that T cells specific for these epitopes are involved in the pathogenesis of viral-induced demyelination. Supported by grants from the USPHS (RO1 NS28752) and (RO1 NS33008).

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IMMUNOSUPPRESSIVE EFFECT EXERTED BY HIGH MOLECULAR WEIGHT COMPONENTS FROM *Ascaris suum* EXTRACT. E.S. Faquim and M.S. Macedo, Inst. Biomedical Sciences, Univ. of S  o Paulo, S  o Paulo, SP, Brazil.

Fractionation of *Ascaris suum* extract (Asc) by gel filtration chromatography results in two peaks (PI and PIII) with distinct molecular weight (MW) and biological activities (Soares et al., Int Arch Allergy Immunol 97: 37, 1992). PI, but not PIII, has a suppressive effect on IgE antibody responses. The present work describes the effect of these peaks on a cell-mediated immune response against ovalbumin (OA). For this, groups of A/Sn mice were immunized with OA, OA+Asc, OA+PI or PIII in CFA in the base of the tail, with the peaks concentrated to the original volume of the extract application in the chromatography column. Hypersensitivity reactions elicited by challenge with aggregated OA were measured on day 8. Groups injected with Asc or PI showed depressed immediate (antibody-mediated) and delayed-type reactions when compared to the group immunized with OA alone or OA+PIII. Proliferation of lymph node cells from these animals, stimulated *in vitro* with OA, was profoundly diminished in Asc and partially diminished in the PI-injected groups. IFN- γ and IL-10 production in these groups were affected in the same way. The proliferative responses of Asc-, PI- or PIII-primed cells to the respective antigens were equally strong and characterized by high levels of IL-10 secretion. However, only cells from OA+PIII-immunized animals, restimulated *in vitro* with PIII, produced IFN- γ and displayed comparatively lower levels of IL-10. These results indicate that the high MW components from the helminth extract do also exhibit a suppressive effect on the cell-mediated immune response. In addition, this effect seems to be related to the cytokine pattern induced by them, but not by the low MW components.

FUNCTIONAL ROLE AND SEQUENCE ANALYSIS OF A LYMPHOCYTE ORPHAN OPIOID RECEPTOR. William P. Halford,^{*} Michael Serou,[†] Bryan M. Gebhardt,^{†‡} and Daniel J.J. Carr^{†‡}. Departments of ^{*}Microbiology and Immunology, [†]Ophthalmology and [‡]Pharmacology, [†]LSU Neuroscience Center; LSU Medical Center, New Orleans, LA 70112-1393

Pharmacologic evidence indicates that lymphocytes express opioid receptors, but this finding has been questioned. By DNA sequencing of reverse transcription-polymerase chain reaction products, we have found that mouse lymphocytes express mRNA encoding an orphan opioid receptor. These mRNA transcripts were detected in the CD4⁺, CD8⁺, and CD4⁺CD8⁺ lymphocyte subpopulations. Northern blot analysis confirmed that splenic lymphocytes express a 1.5 kilobase orphan opioid receptor mRNA. Fifteen bases encoding Tyr⁷¹-Arg⁷⁵ in the first intracellular loop are alternatively spliced, suggesting that orphan opioid receptor mRNA encodes two receptor subtypes. Treatment of lipopolysaccharide-stimulated lymphocytes with orphan opioid receptor antisense oligonucleotides suppressed polyclonal IgG and IgM production by 50%. Our results provide direct evidence that lymphocytes express an opioid-like receptor gene, and suggest that this receptor plays a functional role in immunocompetence.

This work was supported in part by a grant from the Department of the Army, Cooperative Agreement DAMD17-93-V-3013. This work does not necessarily reflect the position or the policy of the government, and no official endorsement should be inferred.

COOPERATIVE AGREEMENT NO.: DAMD17-93-V-3013

TITLE: NEURAL RESPONSE TO INJURY, PREVENTION, PROTECTION, AND
REPAIR

CHAPTER: 6B: NEUROIMMUNOLOGY OF STRESS

INVESTIGATOR: DANIEL J.J. CARR, PH.D., PRINCIPAL INVESTIGATOR

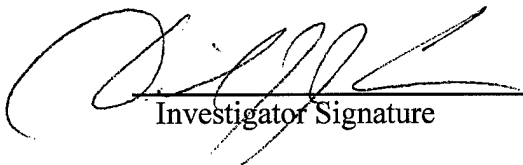
REPORT DATE: AUGUST 15, 1996

TYPE OF REPORT: ANNUAL REPORT : AUGUST 1, 1995 THROUGH JULY, 1996

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

- () Where copyrighted material is quoted, permission has been obtained to use such material.
- () Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.
- (X) Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.
- (X) In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).
- () For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.
- () In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.
- () In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH guidelines for Research Involving Recombinant DNA Molecules.
- (X) In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



Investigator Signature

10-4-96

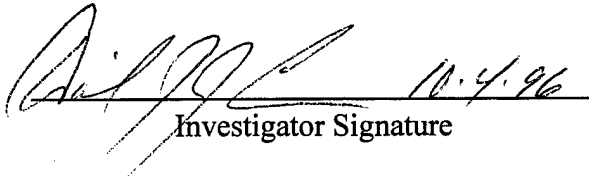
Date

ANIMAL USE
SEPTEMBER 20, 1995 THROUGH JULY, 1996

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: The Neuroimmunology of Stress, Injury, and Infection**, are as follows:

Species	Number Allowed	Number Used	LSU IACUC#
rabbit	2	1	1341
mice	820	700	1341


Investigator Signature

DETAILED BUDGET FOR NEXT BUDGET PERIOD DIRECT COSTS ONLY		FROM 10/96	THROUGH 10/97	GRANT NUMBER DAMD17-93-V-3013			
PERSONNEL (Applicant organization only)		TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED (Omit cents)		
NAME	ROLE ON PROJECT				SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Daniel J.J. Carr	P.I.	12	20	\$49,087	\$ 5,889	\$ 424	\$ 6,313
William P. Halford	GRAD ASST	12	100	\$15,000	\$15,000	\$1,050	\$16,050
SUBTOTALS →					\$20,889	\$1,474	\$22,363
CONSULTANT COSTS							0
EQUIPMENT (Itemize)							0
SUPPLIES (Itemize by category)							
Plasticware/glassware		\$3,000					
Tissue culture		\$2,000					
Chemical (RT-PCR reagents)		\$3,000					
Radionucleotides		\$1,000					
Mice 400 @ \$600/mouse		\$2,400					
Antibodies		\$ 544					
							\$11,944
TRAVEL							
Two people attend one national meeting each (\$780/person)							\$ 1,560
PATIENT CARE COSTS		INPATIENT					0
		OUTPATIENT					0
ALTERATIONS AND RENOVATIONS (Itemize by category)							0
OTHER EXPENSES (Itemize by category)							
Animal care 40 mice @\$0.16/mouse/day		\$2,336					
Publication expenses		\$1,000					
Membership in AAI and ISNIM		\$ 415					\$ 3,751
SUBTOTAL DIRECT COSTS FOR NEXT BUDGET PERIOD							0
CONSORTIUM/CONTRACTUAL COSTS							
DIRECT COSTS \$					TOTAL →		
INDIRECT COSTS \$							0
TOTAL DIRECT COSTS FOR NEXT BUDGET PERIOD (Enter on Page 1, Item 10a) →					\$ 39,618		

BUDGET JUSTIFICATION

GRANT NUMBER

DAMD17-93-V-3013

SUPPLEMENTAL INFORMATION REGARDING *ITEMS* IN THE PROPOSED BUDGET FOR THE NEXT PERIOD WHICH REQUIRE EXPLANATION OR JUSTIFICATION. (See instructions)

PERSONNEL

D.J.J. Carr will be responsible for the design of the studies outlined under plans. He will interpret and report the results of these studies, and will supervise the daily operation of the graduate student. Publications and presentations at scientific meetings of the results from work stemming from this project will be organized under the auspices of the P.I. Based on the previous three years, the P.I. will devote 25% of his time to the project and request 12% of his salary.

W.P. Halford is a fourth year graduate student who is continuing on this project through his graduation and temporary research associate through January, 1997. Bill will pursue the stress-related studies assessing cytokine expression by RT-PCR and ELISA techniques. In addition, he will be responsible for the assessment of viral reactivation using co-culture techniques with CV-1 indicator cells and trigeminal ganglia following hormone agonist and antagonist therapy and hyperthermic application. Finally, the results will be prepared by Mr. Halford and analyzed with the aid of the P.I. The support rendered by this award and the data generated from these studies constitute the bulk of Mr. Halford's dissertation.

**It is anticipated that a new graduate student will join the laboratory this fall and will take over the remainder of the project to completion.

SUPPLIES

The reagents that have been requested are necessary to perform the planned experiments outlined in the progress report summary.

TRAVEL

Current plans are for the P.I. and graduate student to attend and present data generated from this project at the AAI meeting in San Francisco, CA next February, the ISNIM meeting in Bethesda, MD next November, or the PNIRS meeting in Boulder, CO next June.

OTHER EXPENSES

The P.I. predicts that at least 2-3 manuscripts will be forthcoming over the next 6-9 months from the data generated as outlined or from ongoing experiments. On average, each publication is a minimum of \$500 for reprints and/or page charges. Membership in AAI or ISNIM allows the P.I. to receive journals covering the discipline in which this research is conducted at reduced rates as well as register for meetings at reduced costs.

CURRENT BUDGET PERIOD	FROM	THROUGH
	9/20/95	9/20/96

The following pertains to your CURRENT PHS budget. This information may be used in determining the amount of support for the NEXT budget period.

A. CURRENT BUDGET	TOTAL ESTIMATED EXPENDITURES AND OBLIGATIONS (1)	ESTIMATED UNOBLIGATED BALANCE (2)	EXPLAIN ANY SIGNIFICANT ESTIMATED UNOBLIGATED BALANCE IN COLUMN 2 (3)
TOTAL DIRECT COSTS	\$38,342	0	
INDIRECT COSTS (As provided)	\$ 5,937	0	
TOTALS →	\$44,279		

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Daniel J.J. Carr	Assistant Professor

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
UTMB, Dallas, TX	B.S.	1983	Biology
UTMB, Galveston, TX	Ph.D.	1987	Microbiology
UTAB, Birmingham, AL	Post-doc	1987-1988	Neuroimmunology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

PROFESSIONAL EXPERIENCE.

- 1988-1991: Research Assistant Professor, Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, AL.
- 1991-present: Assistant Professor, Department of Microbiology, Immunology, & Parasitology, LSU Medical Center, New Orleans, LA.
- 1993-present: Adjunct Assistant Professor, Department of Pharmacology; Member, LSU Neuroscience and LSU Stanley S. Scott Cancer Center, LSU Medical Center, New Orleans, LA.

HONORS:

- 1985 James W. McLaughlin predoctoral fellowship, UTMB, Galveston, TX.
- 1990 Distinguished Speaker at the II International Symposium on Endocrinology under 35, Siena, Italy.
- 1992 Randall Lecturer, American Society of Microbiology South Central Branch, Starkville, MS.
- 1995 Guest editor, *Advances in Neuroimmunology* issue devoted to signaling transduction..

SELECTED PUBLICATIONS: (Selected from 43 peer-reviewed articles and 30 book chapters or solicited reviews).

1. Halford, W.P., B.M. Gebhardt, & **D.J.J. Carr**. 1996. Persistent cytokine expression in trigeminal ganglion latently infected with herpes simplex virus type 1. *J. Immunol.* in press.
2. **Carr, D.J.J.**, T.J. Rogers, & R.J. Weber. 1996. The relevance of opioids and opioid receptors on immunocompetence and immune homeostasis. *Proc. Soc. Exp. Biol. Med.* in press.
3. Scott, M. & **D.J.J. Carr**. 1996. Morphine suppresses the alloantigen-driven CTL response in a dose-dependent and naltrexone-reversible fashion. *J. Pharmacol. Exp. Ther.* in press.
4. Garza, H.H.Jr., O. Prakash, & **D.J.J. Carr**. 1996. Aberrant regulation of cytokines in HIV-1 TAT₇₂ transgenic mice. *J. Immunol.* 156:3631-3637.
5. Halford, W.P., B.M. Gebhardt, & **D.J.J. Carr**. 1996. Mechanisms of herpes simplex virus type 1 reactivation. *J. Virol.* 70:5051-5060.
6. **Carr, D.J.J.**, M. Scott, L.L. Brockunier, J.R. Bagley & C.P. France. 1996. Mirfentanil antagonizes morphine-induced suppression of splenic natural killer activity. *Immunopharmacol.* in press.
7. **Carr, D.J.J.**, M. Scott, L.L. Brockunier, J.R. Bagley & C.P. France. 1996. The effect of novel opioids on natural killer activity and tumor surveillance *in vivo*. *Adv. Exp. Biol. Med.* in press

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- France, C.P., D.J.J., L.L. Brockunier, & J.R. Bagley. 1995. OHM3597: A novel fentanyl derivative with morphine-like behavioral effects in rhesus monkeys. *Drug Development Res.* 35:49-58.
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- Garza, H.H. & D.J.J. 1995. Interactions of human immunodeficiency virus type 1 transactivator of transcription protein with signal transduction pathways. *Adv. Neuroimmunol.* 5:321-326.
- Halford, W.P. & D.J.J. Carr. 1995. Subversion of intracellular signal transduction by herpes simplex virus type 1. *Adv. Neuroimmunol.* 5:327-334.
- Carr, D.J.J., G. Carpenter, H.H. Garza Jr., O. Prakash, & C.P. France. 1995. Chronic and infrequent opioid exposure suppresses IL-2R expression on rhesus monkey peripheral blood mononuclear cells following stimulation with pokeweed mitogen. *Intern. J. Neurosci.* 81:137-148.
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- Garza, H.H., S. Mayo, W. Bowen, B.R. DeCosta, & D.J.J. Carr. 1993. Characterization of a azidophenazocine-sensitive sigma receptor on splenic lymphocytes. *J. Immunol.* 151:4672-4680.
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- Carr, D.J.J. & C.P. France. 1993. Immune alterations in chronic morphine-treated rhesus monkeys. *Adv. Exp. Med. Biol.* 335:35-40.

OTHER SUPPORT

(Use continuation pages if necessary)

GRANT NUMBER

DAMD17-93-V-3013

FOLLOW INSTRUCTIONS CAREFULLY. Incomplete, inaccurate, or ambiguous information about OTHER SUPPORT could lead to significant delays in the review and/or funding of the application.

Other support is defined as all funds or resources, whether Federal, non-Federal, or institutional, available to the principal investigator/program director (and other key personnel named in the application) in direct support of their research endeavors through research or training grants, cooperative agreement, contracts, fellowships, gifts, prizes, and other means. However, in the case of prizes and gifts, only those that support the specific project must be reported.

Reporting requirements are: For each of the key personnel, describe (1) all currently **active** support and (2) all applications and proposals **pending** review or award, whether related to this application or not. If the support is part of a larger project, identify the principal investigator/program director and provide the data for the relevant subproject(s). If an individual has no active or pending support, check "None." Use continuation pages as needed to provide the required information in the **format** as shown below. Information may be combined as long as the format remains the same. For example, all key personnel who have no other support may be listed on a single page. **DO NOT SEND** in a separate page for each person listed for whom "None" is checked. Key personnel are defined as all individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project.

Name Daniel J.J. Carr Active X Pending None

a. Source and identifying no. NINDS NS35470-01 P.I. CARR

Title THE NEUROIMMUNOLOGY OF HSV-1 REACTIVATION

b. Your role on project P.I. % Effort 30

c. Dates and costs of entire project (For renewals, include only the most recent competitive award. List direct and indirect costs separately.)

DIRECT (\$228,704) INDIRECT (\$83,344)

d. Dates and costs of current year DIRECT (\$93,833) INDIRECT (\$26,699)

e. Specific aims of project Does stress affect chemokine expression in the TG; Are T lymphocytes
required for HSV-1 suppression in reactivation in vivo and in vitro

f. Describe scientific and budgetary overlap There are no scientific overlaps. It should be noted
that the data generated from the support of the current grant was extremely
instrumental in the funding of the NINDS grant.

g. Describe adjustments you will make if the present application is funded (budget, % effort, aims, etc.)

No adjustments are required

PROGRESS REPORT SUMMARY		GRANT NUMBER DAMD17-93-V-3013	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR Dr. Nicolas Bazan (Chapter 6B-Daniel JI Carr, PhD)		PERIOD COVERED BY THIS REPORT	
APPLICANT ORGANIZATION LSU Medical Center		FROM 9/20/95	THROUGH 8/15/96
TITLE OF PROJECT (Repeat title shown in item 1 on first page) NEURAL RESPONSE TO INJURY, PREVENTION, PROTECTION, AND REPAIR; NEUROIMMUNOLOGY OF STRESS (SEE INSTRUCTIONS)			

1. SPECIFIC AIMS

The specific aims of the project are: (1) To determine the effects of a brief period of thermal stress (10 min at 43°C) and restraint stress (60 min) as indirect mediators of HSV-1 reactivation from neural tissues, (2) To determine the neuroendocrine mechanism of stress-induced viral reactivation by measuring corticosterone and monoamine levels in the serum and nervous tissues of latently infected, stressed animals, (3) To determine the effect of stress on antiviral immune responses in mice undergoing viral reactivation, and (4) To determine the effect of exogenous stress hormone agonists and antagonists on stress-induced viral and antiviral immunity.

2. STUDIES AND RESULTS

The following is a summary of each goal (specific aim) emphasizing this past year's accomplishments.

The first goal was to compare stressors on herpes simplex virus type 1 (HSV-1) reactivation using co-culture techniques to verify the production of infectious virus, immunohistochemical techniques for immunopositive staining of viral proteins associated with viral reactivation, and reverse transcription (RT)-polymerase chain reaction (PCR) assays to identify the expression of viral transcripts. The results presented in the first year's progress report (Tables 1 & 2) showed that the heat stress paradigm induced a higher percentage of reactivations (80% or 8/10 mice reactivated) as compared to the restraint stress model (40% or 4/10 mice reactivated) as determined by the recovery of infectious virus in the trigeminal ganglia, corneas, and tear film. This experiment was repeated three times with similar results each time. It was noted that statistical analysis of the data indicated a high degree of significance ($p < .005$) as determined by ANOVA. In mice that were not stressed, no recovery of infectious virus was found in any tissue examined. In both cases, the mice were ocularly infected followed by a 35 day incubation period.

No experiments have thus far been carried out to immunohistochemically define viral reactivation *in vivo*.

We have attempted to use reverse transcriptase (RT)-polymerase chain reaction (PCR) to detect HSV-1 lytic phase transcripts of all three kinetic classes (i.e., immediate-early, delayed-early, and late) as a marker of HSV-1 reactivation following hypothermic stress of latently-infected mice. The HSV-1 specific primers chosen amplify infected cell polypeptide 27 mRNA (i.e., ICP27, immediate early), ribonucleotide reductase mRNA (i.e., RR, delayed-early), and virion protein 23 mRNA (i.e., MP23, late). The ICP27 and VP23 PCR primers have been used before to detect HSV-1 mRNAs during reactivation in a rabbit model (Bloom et al., 1994). However, we have found that aside from a single experiment where ICP27 mRNA was detected

in 2/2 mice (115 days post infection) 12 hours after hyperthermic stress, detection of HSV-1 lytic phase transcripts following hyperthermic stress has been inconsistent (2/16 mice).

One notable observation that has arisen out of efforts to detect HSV-1 lytic phase transcripts as markers of reactivation is the detection of VP23 mRNA in the TG of 16/16 latently-infected mice (i.e., 30-44 days post infection), regardless of stress treatment. The identity of these PCR products has been confirmed by Southern blotting. The possibility that the VP23 PCR product was amplified from contaminating HSV-1 DNA in the RNA preparation was ruled out using interchangeable sense to anti-sense strand primers as well as DNase and RNase treatment of isolated RNA. Currently, emphasis is focused on obtaining the full length transcript using the RACE technique for subsequent cloning and sequencing of the latently expressed transcript.

During the past year, the development of an *in vitro* model to study HSV-1 reactivation has been accomplished (*J. Virol.* 70:5051-5060, 1996.). In addition, the role of cytokines in the potential regulation of heat-stressed induced reactivation in this model system has been studied (Halford, Scott, and Carr, *Virol.*, submitted). The development of this model has allowed us to characterize the hypothesized neuroendocrine molecules that are predicted to influence HSV-1 reactivation and the mechanism by which the hormones initiate such an event as detailed next.

The goal of the second specific aim was to initiate studies in quantitating tissue monoamine and serum corticosterone levels in the stressed mice (infected and uninfected) to determine if a correlation exists between those levels and viral reactivation. In the progress report for year 1 (last year), we presented data measuring serum corticosterone and brainstem monoamine levels 24-hr following hyperthermic stress. Even though this time point is not ideal for such measurements (since neurotransmitter and HPA axis hormone release are rapid responders to stress), the results show that the procedure in isolating and processing tissue allow us to make accurate measurements. We found no differences in the monoamine levels measured (norepinephrine, epinephrine, dopamine, and serotonin) in the brainstem. Serum corticosterone levels were elevated in the stressed mice compared to the non-stressed infected mice. However, there were no differences between serum corticosterone levels between stressed and non-stressed uninfected mice. Potentially, the virally-infected animals which are in a process of viral reactivation may have signalled immune cells to secrete ACTH which could then activate the HPA axis ultimately resulting in an elevation in corticosterone production. Precedence for the involvement of leukocytes in the HPA axis has previously been reported. During the last year, we have investigated the role of the catecholamine (epinephrine) and the synthetic glucocorticoid, dexamethasone in HSV-1 reactivation primarily using the *in vitro* culture system. Whereas epinephrine did not reactivate latent trigeminal ganglion cell cultures, dexamethasone did in a dose-dependent fashion (*J. Virol.* 70:5051-5060, 1996). Moreover, dexamethasone facilitated HSV-1 reactivation in hyperthermically-stressed cultures as evident by the detection of lytic virus earlier in the culture supernates as well as the expression of immediate early gene transcripts in the treated cell cultures. Since dexamethasone appeared to affect HSV-1 reactivation *in vitro*, preliminary studies were conducted *in vivo* using dexamethasone and the glucocorticoid synthesis inhibitor, cyanoketone. Initial studies have shown that using cyanoketone (50-100 mg/kg) to treat mice (24 and 2 hr prior to hyperthermic stress) indicate that the hypothalamic pituitary adrenal (HPA) axis may be involved in hyperthermic-induced reactivation of HSV-1. Specifically, 3/18 or 16.7% of the cyanoketone-treated mice reactivated following hyperthermic stress whereas vehicle-treated controls reactivated in greater frequency; 6/20 or 30%. These results are a

culmination of four independent experiments. Cyanoketone was found to block corticosterone synthesis as indicated in the serum levels of corticosterone (i.e., cyanoketone-treated mice has levels ranging from 14-67 ng/ml versus 764 +/- 30 ng/ml for vehicle-treated mice 1 hr post hyperthermic stress) following stress. Since these initial studies *in vivo* studies along with the previous *in vitro* studies suggested a role for the HPA axis, the reactivation potential for glucocorticoids was investigated *in vivo*. Dexamethasone (1.0 mg/ml) added to the drinking water (*ad libitum* for 24 hrs) was assessed for reactivation in the presence and absence of hyperthermic stress. Dexamethasone alone had no effect on HSV-1 reactivation in latently infected mice (0/8 reactivated) and did not augment the reactivation of hyperthermically-stressed mice (1/9) compared to vehicle-treated, hyperthermically-stressed animals (2/8). Since the stress paradigm is currently eliciting only 25-30% reactivation, we are attempting to improve our stress paradigm in order to increase the reactivation rate of the latent mice.

The goal of the third specific aim was to determine the effect of stress on antiviral immune responses in mice undergoing viral reactivation. Over the past year, studies were conducted to initially measure serum antibody levels as well as cytokine levels (both transcript and protein) in the trigeminal ganglia of mice following initial ocular inoculation from acute (days 5-7) to latent (days 30-135) infection. The results of these studies (Halford, Gebhardt, and Carr, *J. Immunol.* in press) indicate two important findings: (i) mononuclear cell infiltration and selective cytokine gene expression exist during latency (out at least 4 months) and (ii) antibody titers continue to rise following latency. These results suggest that the host is continually exposed to low levels of virus or viral antigen that propagate active T cell cytokine synthesis and clonal expansion of HSV-1 specific B cells. Stress effects on cytokine synthesis and antibody titers are planned during this year.

The fourth goal of this project was to determine the effect of exogenous stress hormone agonists and antagonists on stress-induced viral and antiviral immunity. The hypothesis on which this investigation is based centers on the notion that corticosterone and/or catecholamines are in part responsible for the reactivation of HSV-1 in latently infected mice following hyperthermic stress. Accordingly, the administration of exogenous catecholamines or corticosteroids are predicted to reactivate HSV-1 without subjecting the latently infected animals to the stress paradigm. We have made some progress in the involvement of the HPA axis in stress-induced reactivation of HSV-1 as outlined under specific aim #2 progress. Plans have been made to further these initial observations as outlined below.

3. SIGNIFICANCE

This project is organized around the central hypothesis that stressors (temperature and restraint) have detrimental effects on immunocompetence potentially resulting in the reactivation of latent pathogens. Our working model is a latently-infected herpes simplex virus type-1 (HSV-1) mouse. Our central hypothesis is that the hypothalamic pituitary adrenal (HPA) axis and the sympathetic nervous system (primarily adrenergic pathways) are the parties responsible for HSV-1 reactivation. Alterations in serum corticosterone and shifts in monoamines in the brains, trigeminal ganglia, and brain stems are predicted to correlate to the reactivation status of the stressed mice. In addition, assessing the local immune parameters (specifically cytokine

production) and relating their levels to viral reactivation following stress are predicted to be helpful in identifying and elucidating a(the) mechanism associated with viral reactivation. To this end, we have made significant advances in assessing the degree of latency of virus *in vivo* as well as establish the immune response (both humoral and cell-mediated) to acute and latent virus. We have also developed an *in vitro* model system to study HSV-1 reactivation under pharmacologically controlled conditions. This will allow us to investigate stimulants of viral reactivation at both the cellular and molecular levels. We believe that this is the first time that such a culture condition has been established and should prove useful in future assessment of anti-viral drugs and their mechanism(s) of action at the molecular level. Finally, we have developed stressors that elicit HSV-1 reactivation *in vivo* and *in vitro*. Since we have now established the baseline for immune parameters during latent infection, we are in a position to ascertain the effects of hyperthermic stress on changes that may take place within the confines of the immune system ultimately resulting in controlled viral reactivation. This scenario will allow us to fully utilize pharmacological tools currently available to ascertain the potential of these drugs in blocking reactivation. Such an event is predicted to prove valuable to the goals and mission of this chapter of the grant awarded to the LSU Medical Center by the Department of the Army.

4. PLANS

During the next budget year, we plan to:

A. Conclude goal #3 by applying the synthetic glucocorticoid agonist dexamethasone and the corticosterone synthesis inhibitor cyanoketone in latent mice and assessing these compounds in blocking or potentiating viral reactivation. In addition, we plan on assessing adrenoceptor agonists and antagonists in blocking or eliciting reactivation of HSV-1 in latent mice. These studies will be conducted using a variety of doses and class-selective adrenoceptor ligands.

B. Conclude goal #4 by measuring cytokine transcripts and protein in the trigeminal ganglia by RT-PCR and ELISA respectively following stress over a 30 min to 24 hr time period. Preliminary results suggest IL-6 may play a role in reactivation since IL-6 mRNA levels are transiently elevated at the 12 hr time point post-heat stress whereas TNF- α , IFN- γ , IL-10, and IL-1 β are not.

5. HUMAN SUBJECTS

Not applicable.

6. VERTEBRATE ANIMALS

No change in the protocol is anticipated.

7. PUBLICATIONS RESULTING FROM THIS RESEARCH:

Publications:

Carpenter, G.W., H.H. Garza jr., B.M. Gebhardt, & D.J.J. Carr. 1994. Chronic morphine treatment suppresses CTL-mediated cytotoxicity, granulation, and cAMP responses to alloantigen. *Brain, Behavior, Immun.* 8:185-203.

Carpenter, G.W. & D.J.J. Carr. 1995. Pretreatment with β -funaltrexamine blocks morphine-mediated suppression of CTL activity in alloimmunized mice. *Immunopharmacol.* 29:129-140.

Carr, D.J.J. & G.W. Carpenter. 1995. Morphine-induced suppression of cytotoxic T lymphocyte activity in alloimmunized mice is not mediated through a naltrindole-sensitive delta opioid receptor. *Neuroimmunomodulation.* 2:44-53.

Carr, D.J.J., G.W. Carpenter, H.H. Garza jr., M.L. Baker, & B.M. Gebhardt. 1995. Cellular mechanisms involved in morphine-mediated suppression of CTL activity. *Adv. Exp. Med. Biol.* 373:131-139.

Halford, W.P., B.M. Gebhardt, and D.J.J. Carr. 1995. Functional role and sequence analysis of a lymphocyte orphan opioid receptor. *J. Neuroimmunol.* 59:91-101.

Carpenter, G.W., L. Breeden, & D.J.J. Carr. 1995. Acute exposure to morphine suppresses CTL activity. *Int. J. Immunopharmacol.* 17:1001-1006.

Carr, D.J.J. and M. Serou. 1995. Exogenous and endogenous opioids as biological response modifiers. *Immunopharmacol.* 31:59-71.

Halford, W.P., B.M. Gebhardt, & D.J.J. Carr. 1996. Mechanisms of herpes simplex virus type 1 reactivation. *J. Virol.* 70:5051-6060.

Carr, D.J.J., T.J. Rogers, and R.J. Weber. 1996. The relevance of opioids and opioid receptors on immunocompetence and immune homeostasis. *Proc. Soc. Exp. Biol. Med.* in press.

Halford, W.P., B.M. Gebhardt, & D.J.J. Carr. 1996. Persistent cytokine expression in trigeminal ganglion latently infected with herpes simplex virus type 1. *J. Immunol.* in press.

Carr, D.J.J. 1996. Corticosterone is not associated with acute morphine-mediated suppression of peritoneal cytotoxic T lymphocyte activity in alloimmunized mice. *Drug Alcohol Depend.* submitted.

Halford, W.P., M. Scott, & D.J.J. Carr. 1996. Cytokine expression in HSV-1 latently infected trigeminal ganglion cell cultures. *Virol.* submitted.

Abstracts:

Halford, W.P., B. Gebhardt, and D.J.J. Carr. 1994. HSV-1 latently infected mice display an altered response to stress: Implications for antiviral immunity. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL

Halford, W.P., B.M. Gebhardt, and D.J.J. Carr. 1994. Mouse lymphocytes express an orphan opioid receptor. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL.

Carr, D.J.J., L. Breeden, G.W. Carpenter, & B.M. Gebhardt. 1994. The frequency of exposure to morphine differentially affects CTL activity in alloimmunized mice. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL.

Baker, M.L., D.J.J. Carr, & B.M. Gebhardt. 1994. Morphine suppresses peritoneal and splenic CTL activity in a dose dependent fashion in alloimmunized mice. 6th Psychoneuroimmunology Research Conference, Nov. 17-20, Key Biscayne, FL.

Halford, W.P., M. Serou, B.M. Gebhardt, & D.J.J. Carr. 1995. Functional role and sequence analysis of a lymphocyte orphan opioid receptor. CPDD Meeting, June 10-15, Scottsdale, AZ

Halford, W.P., B.M. Gebhardt, and D.J.J. Carr. 1995. Analysis of the immune response during stress-induced reactivation of herpes simplex virus type 1. 9th International Congress of Immunology, July 23-29, San Francisco, CA.

Carr, D.J.J. and W.P. Halford. 1996. Lymphocytes delay kinetics of HSV-1 reactivation from *in vitro* explants of latently infected trigeminal ganglia (TG). 3rd International Congress of the International Society for Neuroimmunomodulation, Nov. 13-15, Bethesda, MD.

8. INVENTIONS AND PATENTS

Not applicable.

PROGRESS REPORT (Personnel and Study Subjects)

GRANT NUMBER

DAMD17-V-93-3013

All Personnel for the Current Budget Period
and Any Planned Changes in Personnel for the Next Budget Period

Use two sections. In the first section list *All Current Personnel*. In the second section list *Planned Personnel Changes*.

Name	Degree(s)		Role on Project (e.g., PI, Res. Assoc.)		Annual % Effort
Daniel J.J. Carr	PHD		PI		12
William P. Halford	MS		GRAD ASST		100
		PII Redacted			

Provide the number of subjects enrolled in the study *to date* (cumulatively since the most recent competitive award) according to the following categories. (See Page 8 for definition.)

	American Indian or Alaskan Native	Asian or Pacific Islander	Black, not of Hispanic Origin	Hispanic	White, not of Hispanic Origin	Other or Unknown	TOTAL
Female							
Male							
Unknown							
TOTAL							



ELSEVIER

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Immunopharmacology

Exogenous and endogenous opioids as biological response modifiers

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Abstract

Narcotic opioid compounds are among the most widely prescribed drug interventions for individuals suffering pain. Among the unwarranted side effects of respiratory depression, constipation, and physical dependence are the immunosuppressive qualities, particularly those which affect cell-mediated immunity. The immunosuppressive characteristics of opioid narcotics (e.g., morphine) have recently come into focus with the advent of acquired immune deficiency syndrome (AIDS) and the putative causative agent, human immunodeficiency virus type 1 (HIV-1). Specifically, a vast reservoir of HIV-1-infected individuals exists among drug abusers. Moreover, experimental evidence would suggest narcotic opioids may increase viral load in infected individuals by modifying the cellular machinery of activated leukocytes. Likewise, investigators have shown that opioids modify tumor growth and development. In this review, a comparison between endogenous opioid peptides and exogenous opiates on cell-mediated immunity and its relationship to viral infection and tumors is described.

Keywords: Natural killer activity; Cytotoxic T lymphocyte; Morphine; Fentanyl; Cancer; AIDS

1. Introduction

The modern concept that cells of the immune system (granulocytes, lymphocytes, and

monocytes/macrophages) can specifically respond to opioids (e.g., β -endorphin or morphine) was born out of original observations showing aberrant immunologic patterns and responses in heroin abusers (Dismukes et al., 1968; Louria, 1974; Brown et al., 1974) and morphine-treated mice (Lefkowitz and Chiang, 1975). Coupled with these studies were the initial reports suggesting lymphocytes possessed binding sites for exogenous opioids (e.g., morphine) and endogenous opioid peptides (e.g., β -endorphin) (Hazum et al., 1979; Wybran et al., 1979; McDonough et al., 1980). Likewise, early in vitro functional studies demonstrated opioid peptides could modify lymphocyte production of antigen-specific

Abbreviations: AIDS, acquired immunodeficiency syndrome; CRH, corticotropin releasing hormone (CRH); CTL, cytotoxic T lymphocyte; DAGO, [D-Ala², N-Me-Phe⁴, Gly-ol⁵]enkephalin; DPDPE, [D-Pen^{2,5}]enkephalin; HIV, human immunodeficiency virus; HPA, hypothalamic pituitary adrenal; IL, interleukin; MHC, major histocompatibility complex; NK, natural killer; PBMC, peripheral blood mononuclear cell; IFN, interferon; SIV, simian immunodeficiency virus

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antibody (Johnson et al., 1982) and lymphocyte proliferative responses to mitogens (Gilman et al., 1982; McCain et al., 1982). Collectively, the data suggest that opioid compounds can appropriately be classified as biological response modifiers; that is, substances that may direct or restore normal immune defenses of the body. Ironically, the administration of opioid narcotics (e.g., morphine and heroin) in vivo tends to suppress the immune system while the addition of opioid peptides (e.g., endogenous peptides including endorphins, enkephalins, and dynorphins) in vitro can augment or suppress cellular and humoral immunity. This review will comment on the data generated in the last decade relative to opioid-mediated immunomodulation of cytolytic effector cells (natural killer [NK] cells and cytotoxic T lymphocytes [CTLs]), potential mechanisms and pathways involved, and the application opioids may have in clinical intervention of disease processes with emphasis on cancer and viral infections.

2. Characterization of opioid peptide-mediated immunoregulation

2.1. Opioid peptide immunomodulatory characteristics

NK cells are a heterogeneous group of leukocytes that are able to kill a variety of normal, virus-infected, and tumor-derived cells in the absence of previous sensitizations, and in a non-major histocompatibility complex (MHC)-restricted fashion. Initial studies suggested that incubation of human peripheral blood mononuclear cells (PBMCs) with β -endorphin (10^{-6} – 10^{-14} M) or [met]enkephalin (10^{-6} – 10^{-9} M) but not [leu]enkephalin (10^{-6} – 10^{-12} M), α -endorphin (10^{-6} – 10^{-12} M), or morphine (10^{-6} – 10^{-12} M) in vitro augmented NK activity in a naloxone-sensitive manner (Mathews et al., 1983). Data were also presented showing β -endorphin increased conjugate formation between effector cells and their targets as well as increase the active number of NK cells (those cells which can lyse the targets following conjugation). These results were confirmed and extended by others in both human and rodent in vitro studies using peripheral blood and

splenic lymphocyte populations (Kay et al., 1984; Mandler et al., 1986; Oleson and Johnson, 1988). However, these and other groups have also reported variable effects of β -endorphin on NK activity in vitro implicating the effects are donor-dependent (Prete et al., 1986; De Sanctis et al., 1986).

The in vitro studies indicate the opioid peptide-mediated effects are direct on the NK cells. Specifically, the enhancing effects are observed within 4–6 h post exposure (taking into account pre-incubation and the 4-h microcytotoxicity assay) which would seemingly rule out the involvement of cytokines such as interferon (IFN)- γ and interleukin-2 known to augment NK activity (Herberman and Ortaldo, 1981). Furthermore, one study showed that antibody to IFN did not block β -endorphin-mediated augmentation of NK activity by large granular lymphocytes (the cells that predominantly mediate NK activity) suggesting endogenous IFN levels are not involved in the opioid peptide-driven event (Mandler et al., 1986).

Since the immunomodulation of the opioid peptides on NK cell activity is rapid, changes in the early events associated with cell activation are most likely modified by the opioids and therefore, influence cytolytic activity. Previous studies have shown opioid peptides increase Ca^{2+} influx or uptake rapidly by murine T lymphocytes (Radulescu et al., 1991) and the Jurkat T cell line (Hough et al., 1990) in a naloxone-sensitive fashion. Since Ca^{2+} is required for the release and execution of perforin (Liu et al., 1995) and granzyme A (Smyth and Trapani, 1995) during the lytic process elicited by most cytolytic effector cells, these observations offer a potential mechanism by which opioid peptides modulate NK activity. Future studies are required to assess the action of opioid peptides on intracellular Ca^{2+} concentrations and mobilization in NK cells themselves. Given that Ca^{2+} pools are potentially located throughout the cell with different release properties (Clapham, 1995), single cell analysis using molecular approaches including confocal microscopy and image quantitative analysis seem warranted to address this issue.

CTLs are another important population of effector cells that monitor tumor and viral pathogenesis in a MHC class I-restricted recognition of antigen (Berke, 1994). Consistent with the effects on murine NK

activity shown one-week sensitivity to this intimate (Hefer (Bessl Conse lowing respon CTLs Howe report CTLs The d ies m Specif using mice more quent opioid cultur ies ut not be in ass or act Th tions occur circul of NK fact. level dorph posses crease zanic levels (Moz of β - be ap (Moz given levels has s degra

activity, β -endorphin and [met]enkephalin have been shown to augment the generation of CTLs in murine one-way mixed lymphocyte cultures in a naloxone-sensitive fashion (Carr and Klimpel, 1986). Related to this observation, the production of IL-2 which is intimately involved in the differentiation of CTLs (Hefeneider et al., 1983) is enhanced by β -endorphin (Bessler et al., 1990; Van Den Bergh et al., 1991). Consequently, the elevation in IL-2 production following exposure to β -endorphin may in part be responsible for the enhancement in the generation of CTLs in the murine mixed lymphocyte cultures. However, using human lymphocytes, other studies report varied effects on the generation and activity of CTLs (Prete et al., 1986; Chiappelli et al., 1991). The differences between the murine and human studies may reside in the source of the lymphocytes. Specifically, the murine investigation was carried out using the inbred C57BL/6 and DBA/2 strains of mice rather than outbred strains which would be more consistent with the human studies. Consequently, the variability in the effects of endogenous opioid peptides in the human mixed lymphocyte cultures may be donor-dependent and therefore, studies utilizing murine inbred mouse lymphocytes may not be an appropriate comparison to the human host in assessing opioid peptide effects in the generation or activity of CTLs.

The physiological significance of these observations suggests that elevated levels of the naturally occurring opioid peptides (e.g., β -endorphin) in the circulation might predictably mirror increased levels of NK or CTL activity mediated by leukocytes. In fact, two studies have shown a correlation in the level of NK activity by PBMCs and serum β -endorphin concentration. Specifically, individuals who possessed 'high' NK activity also exhibited increased β -endorphin levels (Levy et al., 1991; Mozzanica et al., 1991). In contrast, [met]enkephalin levels were inversely correlated with NK activity (Mozzanica et al., 1991). Normal circulating levels of β -endorphin and [met]enkephalin are reported to be approximately 20 and 35 pg/ml respectively (Mozzanica et al., 1991). Although no explanation is given to the inverse relationship between circulating levels of enkephalin and NK activity, a recent report has shown NK cells possess a rapid enzymatic degradation pathway hydrolyzing the Y residue of

the enkephalin peptide (Amoscato et al., 1993) which would alter the efficacy of these opioid peptides on NK activity. Likewise, the identification of several lymphoid cell surface antigens as ectopeptidases has led one group of investigators to assess the metabolism of enkephalins by hematopoietic cells. Purified CD4⁺ and CD8⁺ splenic T lymphocytes have been shown to metabolize [met]enkephalin at a rate of 1.5-2.0 nmol/h/10⁶ cells (Miller et al., 1994a) while resident macrophages hydrolyze [met]enkephalin at a rate of 4.1 ± 1.7 nmol/h/10⁶ cells (Miller et al., 1994b). The activation of the resting cells by anti-CD3 antibody (lymphocytes) or thioglycolate (macrophages) increased the ectopeptidase activity. Consequently, the functional activity of exogenously administered opioid peptides ([met]enkephalin or β -endorphin) in vivo or in vitro would be short-lived.

The observation that opioid peptides augment cytolytic effector cell activity in vitro as well as (in some cases) parallel levels observed in vivo has led to studies evaluating the therapeutic potential of these naturally occurring peptides in individuals with pathogenic manifestations typically monitored by NK cells and CTLs. In one instance, the administration of [met]enkephalin (10 μ g/kg every 48 h over 12 weeks, intravenously) to patients infected with human immunodeficiency virus (HIV) showed no clinically efficacious effect (although transient changes at various times were reported) based on a number of immune parameters measured including T lymphocyte responses to mitogens and antigens, interleukin (IL)-2 production, IL-2 receptor expression, delayed hypersensitivity responses, and changes in phenotypic markers (Zunich and Kirkpatrick, 1988). However, another group has reported the continuous administration of [met]enkephalin (20 μ g/kg, every 48-72 h over a 4 month period) to HIV-infected individuals improves some immunologic parameters including mitogen-stimulated blastogenesis, NK activity, and the percentages of T cell subset populations (Wybran and Plotnikoff, 1991). More recently, the combination of [met]enkephalin (3.0 mg/kg/dose over 1, 3 and 5 day cycles) and AZT (7-15 mg/kg/dose over 1, 3 and 5 day cycles) has been shown to significantly improve the survival of Friend virus leukemia-infected mice (Specter et al., 1994). Unlike the peripheral administration of opioid

peptides, intracerebral inoculation of opioid peptides (i.e., β -endorphin) has been shown to exacerbate viral-mediated death in a mouse model using vesicular stomatitis virus through a T lymphocyte-dependent and naloxone-reversible mechanism (Coons et al., 1991). Based on these findings, the site of action (peripheral versus central) of the opioid peptide following in vivo administration may ultimately influence the immune response as well as viral infection. For example, one might envision opioid peptide interaction with brain opioid receptors leads to suppression of cytolytic effector cell activity while opioid peptide ligation to leukocyte receptors potentiates cytolytic activity.

Endogenous opioid peptides have also been reported to modify tumor progression. Specifically, the administration of [met]enkephalin (30.0 mg/kg, s.c.) has been shown to increase survival time of mice inoculated with L1210 leukemia cells (Plotnikoff et al., 1985) and reduce experimental metastasis of B-16 melanoma cells in C57BL/6 mice (Faith and Murgo, 1988). The reduction in metastasis in enkephalin-treated mice correlated with an elevation in splenic NK activity. Similarly, the enkephalin derivative, [met]enkephalinamide (50 μ g/kg s.c. daily over 7 days) and [met]enkephalin (50-250 μ g/kg s.c. daily over 7 days) suppressed PYB6 fibrosarcoma tumor growth in B6C3F1 mice in a naloxone-reversible manner (Srisuchart et al., 1989). However, the regulation of PYB6 tumor growth following in vivo administration of the enkephalins did not correlate with an elevation in splenic NK activity nor did the tumor cells possess opioid receptors as determined using [3 H]etorphine binding. The administration of β -endorphin (5 nM) has also been shown to augment splenic NK activity in both nude mice and nude mice harboring human ovarian carcinoma cells (Kikuchi et al., 1990). Other studies suggest some tumor cell lines including melanoma (B16-C3 and BL6), neuroblastoma (S20Y), hepatoma (Morris), and mammary (R3230AC) cell lines: (i) display specific opioid binding sites for δ and κ ligands as determined by [3 H]-[D-Ala², D-Leu⁵]enkephalin and ethylketocyclazocine radioreceptor assays (Zagon et al., 1987) and (ii) possess detectable levels of the endogenous opioid peptides [met]enkephalin and β -endorphin (Zagon et al., 1987). In one study, the growth of S20Y neuroblastoma cells

in BALB/c nude mice was dramatically reduced following naltrexone (0.1 mg/kg s.c. daily for 60-70 days) administration suggesting some tumor cell lines may use endogenous opioid peptides in a classical autocrine growth cycle (Zagon and McLaughlin, 1987). The studies above suggest that opioid peptides have a close association in tumor progression either directly through promoting the growth of certain tumor cells (predominately neural-derived) or indirectly through interacting with lymphocytes which monitor tumor growth or interacting with neural opioid receptors which can then modulate the immune response. Moreover, it has been predicted that opioid peptides will serve as useful markers of neuroendocrine differentiation of many tumors including pheochromocytomas, specific carcinomas, pituitary adenomas, carcinoid tumors and paragangliomas (Bostwick et al., 1987).

3. Characterization of opioid narcotic-mediated immunoregulation

3.1. Opioid narcotic immunomodulatory characteristics

As reported above, in vitro and in vivo investigations show that opioid peptides augment NK and CTL activity. Moreover, these effects are blocked by opioid receptor antagonists (e.g., naltrexone) suggesting such immunomodulatory characteristics are mediated via opioid receptors. Unlike opioid peptide action on NK and CTL activity, opioid narcotics (e.g., morphine) are potentially immunosuppressive compounds when administered in vivo. The original observations stemming from work investigating a form of footshock stress (which was known to induce opioid-elicited analgesia) was reported to suppress rodent splenic NK activity through a naltrexone-sensitive pathway (Shavit et al., 1984, 1986a). Similarly, morphine (30-50 mg/kg, s.c.) administered daily over 4 days was found to suppress splenic NK activity in a dose-dependent and naltrexone-reversible fashion (Shavit et al., 1984; Shavit et al., 1986a). Likewise, acute morphine (25 mg/kg, s.c.) administration in rodents was found to suppress splenic NK activity (Bayer et al., 1990). The acute

peripheral (s.c.) administration of *N*-methylnorphine (40.0 mg/kg, a dose which was found to inhibit intestinal motility to the same degree as 30.0 mg/kg morphine), a morphine analogue that does not pass the blood-brain barrier, had no effect on splenic NK activity (Shavit et al., 1986b). In this same study, it was also shown that an acute dose of morphine (10-40 μ g) administered intracerebroventricularly (i.c.v.) could significantly suppress splenic NK activity in a naltrexone reversible manner. Taken together, the results suggested that morphine acted through opioid receptors found centrally (i.e., brain) which subsequently modify, by indirect means, the immune system. This concept was supported by work showing the administration of morphine (6.6 nmol) into the periaqueductal gray matter but not the dorsal hippocampus, medial thalamus, medial amygdala, arcuate nucleus, or anterior hypothalamus led to significant suppression in splenic NK activity (Weber and Pert, 1989). Likewise, the administration (i.c.v.) of [D-Ala², N-Me-Phe⁴, Gly-ol⁵]enkephalin (DAGO, μ -selective peptide) but not [D-Pen^{2,5}]enkephalin (DPDPE, δ -selective peptide) or (-)-(1S,2S)-U50,488 (κ -selective agonist) suppressed splenic NK activity indicating the role of brain μ opioid receptors in suppression of splenic NK activity (Band et al., 1992). The involvement of μ but not δ or κ opioid receptors in morphine-mediated suppression of splenic NK activity in vivo was also supported using the selective δ (naltrindole), κ (norbinaltorphimine), and μ (β -funaltrexamine and naloxonazine) antagonists at doses which block selective opioid agonist-induced analgesia (Carr et al., 1993).

Studies in human subjects have been restricted to parenteral heroin abusers which show a dramatic drop in NK activity (Novick et al., 1989). A pharmacologically controlled study using rhesus monkeys exposed to morphine (3.2 mg/kg daily) over a three year period supported the findings by Kreek's lab. Specifically, monkeys exposed to morphine daily over three years showed a significant decrease (35-40%) in PBMC NK activity that also correlated with a substantial decrease (50%) in the percentage of effector cells (defined as CD8⁺ CD16⁺) in the circulation (Carr and France, 1993).

In the rodent model, daily exposure to morphine (30-50 mg/kg) over 11-14 days has no effect on

splenic NK activity implying a tolerant state has developed to the effects of morphine (Shavit et al., 1986a; Carpenter et al., 1994). However, neither study presented data showing the mice were tolerant to morphine as determined measuring analgesia or some other neural indices. Unlike the lack of an effect following chronic morphine exposure on NK activity, the daily administration of morphine (50.0 mg/kg daily over 11 days) has been shown to suppress CTL activity in alloimmunized C3H/HeN mice (Carpenter et al., 1994; Carpenter and Carr, 1995). Morphine (10^{-6} - 10^{-12} M) had no direct effect on CTL activity when applied during in vitro mixed lymphocyte cultures suggesting that morphine modifies CTL activity by indirect means (Carr and Carpenter, 1995). The subchronic administration of morphine (50.0 mg/kg daily over 5 days) has been shown to have no effect on splenic or peritoneal NK or CTL activity in alloimmunized C3H/HeN mice while the acute exposure to morphine (50 mg/kg once 2 h prior to alloimmunization) has been shown to suppress peritoneal CTL activity (Carr et al., submitted). Consequently, the frequency of exposure to morphine differentially affects CTL and NK activity in C3H/HeN mice.

The duration and frequency of exposure to opioids may be important corollaries to consider when assessing immunomodulation as well (Bryant et al., 1988). Likewise, the sensitivity of the host or strain of animal to morphine is another important consideration when evaluating immune parameters. For example, a previous study has shown different mouse strains are susceptible to varying degrees to the immunosuppressive effects of morphine on antibody production (Bussiere et al., 1992). Similar findings have been demonstrated measuring NK and CTL activity. Specifically, whereas in alloimmunized C3H/HeN mice subchronic exposure to morphine (50 mg/kg daily over 5 days) has no effect on CTL or NK activity, subchronic exposure to morphine (50 mg/kg) in alloimmunized ICR swiss significantly suppressed splenic NK and CTL activity (Carr et al., manuscript in preparation). Recent studies have now focused on pathways (at the systemic and cellular levels) associated with immunosuppression following morphine administration in the hopes of identifying those systems directly responsible as well as the level (peripheral versus central) of involvement.

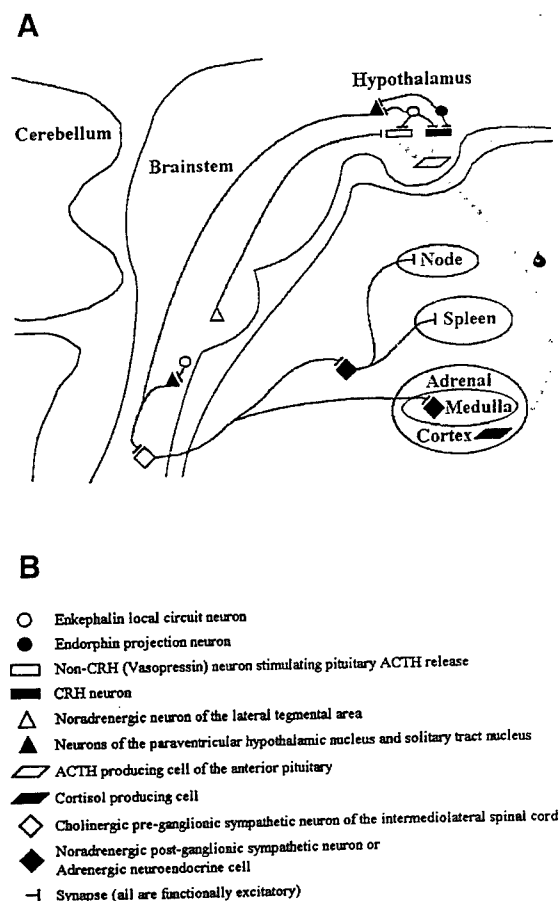
3.2. Mechanisms of opioid alkaloid-induced immunosuppression

Opioid involvement in neurotransmitter release has been documented following the intracisternal administration of morphine (Van Loon et al., 1981; Appel et al., 1986). In addition, opioids have also been shown to influence the hypothalamic-pituitary-adrenal (HPA) axis (Morley, 1981). For example, in rats opiates stimulate growth hormone, prolactin, and adrenocorticotropin hormone release through dopaminergic and/or serotonergic mechanisms. Whereas opiate-mediated stimulation of prolactin release appears to be mediated by opioid receptors outside of the blood-brain barrier, growth hormone stimulation is mediated by opioid receptors located centrally (Simpkins et al., 1991). The effects of opioids on the HPA axis are more discernable following acute administration since it has been shown that subchronic to chronic administration (every 12 h for 4 days) of the μ - and δ -selective opioid peptides DAGO and DPDPE respectively, resulted in the

development of tolerance by the HPA axis as determined measuring plasma corticosterone (Gonzalez et al., 1991).

The HPA axis has also been implicated in immunosuppression of lymphocyte proliferation and spleen and thymic hypoplasia following morphine pellet implantation (Bryant et al., 1990; Bryant et al., 1991; Sei et al., 1991a). More recently, corticosterone involvement in suppression of NK activity following morphine exposure was reported (Freier and Fuchs, 1994). However, corticosterone most probably does not play a significant role in morphine-induced suppression of alloantigen-generated CTLs. Specifically, a recent report showed that only when mice were exposed to corticosterone (16 mg/kg/day) 4 days prior to alloimmunization was

Fig. 1. We hypothesize that the immunomodulatory actions of opioids in the central nervous system are mediated in part by the hypothalamic-pituitary-adrenal axis and the sympathetic branch of the autonomic nervous system. Either enkephalin producing local-circuit neurons or endorphin producing projection neurons may act via μ or κ opioid receptors to stimulate release of corticotropin-releasing hormone (CRH), and a non-CRH, adrenocorticotropin-stimulatory pathway (putatively vasopressin) from hypothalamic neurons. The non-CRH neurons also respond to excitatory inputs from noradrenergic neurons of the ventral tegmental area via α_1 adrenoreceptors, and thus are a potential site of a post-synaptic interaction between adrenergic and opioidergic mechanisms. Adrenocorticotropin released from the anterior pituitary induces release of the immunosuppressant steroid corticosterone from the adrenal cortex. Neurons of the paraventricular nucleus of the hypothalamus and the solitary tract nucleus are stimulated by μ opioid agonists (e.g., morphine), possibly via inhibition of tonically inhibitory interneurons. Paraventricular nucleus and solitary tract nucleus neurons in turn send excitatory projections to preganglionic sympathetic neurons of the intermediolateral cell column of the spinal cord (levels T1-L2). Cholinergic intermediolateral neurons send excitatory efferents to both postganglionic sympathetic neurons, which release norepinephrine at stromal and parenchymal sites in spleen, lymph nodes, bone marrow, and gut-associated lymphoid tissue, and adrenal medullary neuroendocrine cells, which release epinephrine into circulation. Norepinephrine and epinephrine may suppress the immune system by direct or indirect mechanisms at peripheral sites.



any noticeable effect observed in CTL activity (De-Krey and Kerkvliet, 1995). Collectively, the data suggests that corticosterone is at least partially involved in the suppression of splenic NK activity following acute morphine exposure yet, may be less involved in immunosuppression during chronic morphine exposure.

Other studies suggest the involvement of adrenergic pathways in morphine-mediated suppression of NK activity. Specifically, preadministration of mice with α_1 - or β -adrenergic antagonists was found to block morphine-induced suppression of splenic NK activity in a dose-dependent fashion (Carr et al., 1993). In addition, acute morphine administration has been shown to alter the expression of β -adrenergic receptors on both T and B splenic lymphocytes (Baddley et al., 1993). Moreover, adrenergic involvement in morphine-mediated suppression reportedly occurs centrally (Carr et al., 1994a) and may involve the release of corticotropin releasing hormone (Buckingham and Cooper, 1984) which has previously been shown to suppress splenic NK activity following lateral ventricle administration (Irwin et al., 1990). Serotonin whose levels rise in the spleen following acute morphine (50.0 mg/kg) administration (Carr et al., 1994a) may also play a role in the immunosuppression since recent results show serotonin impairs NK activity (Garssadi et al., 1993). Whether adrenergic pathways are also involved in morphine-mediated suppression of CTL activity has not been determined. Pathways which are predicted to be altered following morphine administration are summarized in Fig. 1.

At the cellular level, morphine administration in vivo has been shown to suppress conjugate formation between NK effector and target cells as well as reduce the number of active killer cells (Carr et al., 1994a). Contrary to the effects on NK cells, morphine administration in vivo does not modify the conjugate capacity of CD8⁺ CTL effector cells but rather alters the cytolytic machinery including serine esterase content and release as well as cAMP production following antigen stimulation (Carpenter et al., 1994). An increase in intracellular cAMP levels have been shown to be associated with termination of attack after the interaction of the effector cell with its target (Valitutti et al., 1993). In addition, the signal has been hypothesized to facilitate detachment

from the target, initiate recycling, and preserve granule content (Valitutti et al., 1993). Accordingly, chronic morphine treatment resulting in an aberrant cAMP response in the effector cell population as well as a decrease in serine esterase content is consistent with a reduction in cytolytic effector cell function by CTLs. In addition, previous studies have shown morphine administration in vivo suppresses calcium mobilization (Sei et al., 1991b) and selective cytokine (IL-6 and interferon- γ) production (Bussiere et al., 1993). Consequently, these molecules which are central to the mechanics of CTL-directed cytotoxicity and clonal expansion of effector cells may, in part, explain the immunoregulatory properties of morphine on CTL activity. In vitro, morphine has been shown to suppress IL-2/IL-4 production at high doses ($IC_{50} = 0.8$ mM) (Jessop and Taplits, 1991) yet elevate transforming growth factor- β production at significantly lower doses (0.001–10.0 nM) (Chao et al., 1992). Whereas the effects of morphine on IL-2/IL-4 production were not sensitive to naloxone, morphine-mediated augmentation of transforming growth factor- β was antagonized by pretreatment with naloxone. It has been hypothesized that the augmentation in transforming growth factor- β production by morphine may also contribute to the immunosuppressive characteristics of morphine on cell-mediated immunity (Chao et al., 1992).

Relative to NK cells, acute morphine administration has been shown to modestly reduce the expression of the 2B4 antigen on NK1.1⁺ cells (Carr et al., 1994a). The 2B4 antigen has been predicted to be a component of a signal-transducing complex that may be important for initiating cytotoxicity of target cells (Garni-Wagner et al., 1993). More recently, another surface antigen, CD69 a member of the C-type lectin family that is expressed by NK cells (Ziegler et al., 1994), has been proposed to represent a surface triggering molecule that stimulates the cytolytic machinery of NK cells through protein tyrosine kinases and protein kinase C (Borrego et al., 1993). Recently, a fentanyl-related compound, OHM3295 has been shown to augment splenic NK activity through a μ -opioid receptor-sensitive pathway (Baker et al., 1995). NK1.1⁺ effector cells from OHM3295-treated mice display a significant increase in CD69 antigen following incubation with target cells compared to vehicle-treated mice (Baker et al., 1995). It is tempt-

ing to speculate that the increase or decrease in NK activity following acute administration of OHM3295 or morphine respectively may be related to the expression of CD69 and by inference, tyrosine kinase and protein kinase C activity. However, the relationship between morphine and CD69 expression on NK cells has not been established nor has there been a kinetics study on CD69 expression on NK cells obtained from OHM3295-treated mice. Until such studies are carried out, there is no clear indication that opiates modify this triggering mechanism.

The chronic administration of opioid narcotics could potentially alter the production and expression of endogenous opioid peptides including the endorphins and enkephalins (Bhargava et al., 1989) which are known to be produced and presumably stored by cells of the immune system (Smith and Blalock, 1981; Lolait et al., 1984; Zurawski et al., 1986; Rosen et al., 1989; Linner et al., 1991) in the range of 5-20 pg/mg weight wet of tissue (Sacerdote et al., 1991; Jessop et al., 1994) and are functional for local antinociception (Stein et al., 1990) and possibly NK activity (Carr et al., 1990). However, in a recent study, the expression of the splenic lymphocyte pro-opiomelanocortin transcripts (encodes endorphins) was not found to correlate with chronic morphine exposure (Carpenter et al., 1994). Moreover, the absence of immunoreactive β -endorphin in the spleen in either the vehicle- or chronic morphine-treated mice suggests the endogenous opioid peptide does not play a significant role in the immunomodulatory effects of morphine within the microenvironment of the spleen (Carpenter et al., 1994). Collectively, it would seem that additional studies are warranted to assess the potential relationship between lymphocyte production of endogenous opioid peptides and opioid narcotic exposure.

3.3. The relationship between opioid narcotics and resistance to viruses and cancer

Morphine and fentanyl (oral or transdermal administration) are prescribed narcotics used to treat pain in a variety of patients including those with cancer (Hanks et al., 1987; Portenoy et al., 1993; Twycross, 1982). Similar to morphine, fentanyl has also been shown to suppress NK cell activity (Beilin et al., 1989). Since NK cells have been suggested to

be an important cell population in tumor surveillance (Heberman and Ortaldo, 1981), it is tempting to speculate that opioid alkaloids (e.g., morphine and fentanyl) would decrease the host resistance to tumor growth and development. To this end, it has recently been shown that morphine (10.0 mg/kg) enhances the growth of a variety of tumor cell lines in vivo (Ishikawa et al., 1993). However, other studies suggest opioids (morphine, methadone, or bremazocine) may act to reduce the growth of tumor (adenocarcinoma and squamous) cell lines in vivo (Reubi, 1985) or in vitro (Maneckjee and Minna, 1992). Although these studies seem to contradict one another, the site of administration of the opioid narcotic (e.g., morphine) seems to play a significant role in the outcome. Specifically, intrathecal administration has been found to have deleterious effects on the host immune system or promoting tumor burden while oral or subcutaneous routes have been found to have beneficial effects (Provinciali et al., 1991; Yeager and Colacchio, 1991). Moreover, the dose of drug may also play a role since a recent finding shows low (10.0 mg/kg, s.c.) and high (100.0 mg/kg, s.c.) doses of morphine have no deleterious effects on NK activity (for example) while intermediate doses (25.0-56.0 mg/kg, s.c.) are immunosuppressive (Carr et al., 1994b). Collectively, the data suggest a relationship exists between opioid narcotics and tumor development and growth. However, serious consideration in addressing the types (δ , κ , or μ) of opioid receptors (Pasternak, 1993) potentially involved in association with the immune response (specifically cell-mediated immunity) as it relates to tumor growth have not been undertaken.

Studies that show the analgesic qualities of morphine contribute to the control of tumor metastasis following surgery (Page et al., 1994) seem to reinforce the efficacious qualities of narcotic opioids in alleviating tumor burden in experimental animals and man. Consequently, it would seem that opioids which induce an analgesic state without eliciting immunosuppression may have the most promising attributes for cancer patients. Recently, one study using mice has shown that a fentanyl-related compound OHM3295 induces analgesia comparable to morphine in a naltrexone-reversible manner but unlike morphine or fentanyl, OHM3295 enhanced splenic NK activity (Carr et al., 1994c). Currently,

this compound and other fentanyl derivatives are under evaluation for their potential efficacious effects in tumor burden mice.

A relationship between parenteral drug abuse and the incidence of viral infections has been known for some time (Dismukes et al., 1968). Experimental animal models have also demonstrated that exposure to morphine exacerbates viral infections (Lorenzo et al., 1987; Starec et al., 1991; Carpenter et al., 1994) or in certain instances, elicits protective effects (Veyries et al., 1995). The most provocative findings to date are the reports that show morphine or heroin promote the growth of human immunodeficiency virus type 1 (HIV-1) in PBMCs (Peterson et al., 1990; Adler et al., 1993) and promonocyte/brain cell cocultures (Peterson et al., 1994). Moreover, in microglial/promonocytic cell cocultures, tumor necrosis factor- α has been shown to promote HIV-1 expression following morphine treatment (Chao et al., 1994). Similar results have been reported using simian immunodeficiency virus (SIV) and morphine (Chuang et al., 1993). Although the exact mechanism(s) for the promotion of HIV/SIV replication in morphine-treated cells is unknown, a recent study suggests chronic morphine exposure in vivo increases NF κ B levels in PBMCs following activation (Carr et al., 1995). Since NF κ B binds to the 11-base pair tandem repeat in HIV-long terminal repeat (Nabel and Baltimore, 1987), it seems likely that changes in the levels of transcriptional regulatory elements that promote viral replication as well as act as transcriptional enhancers of genes of immunological relevance may in part explain the results showing narcotic opioids increase HIV/SIV replication. Relative to monkey studies, chronic morphine exposure (3 years) has been shown to increase the percentage of CD4⁺CD29⁺ PBMCs (Carr and France, 1993) which may act as reservoirs for SIV (Murphy-Corb et al., 1989). Given the potential that morphine and heroin are cofactors in HIV-1 disease progression through their immunosuppressive and viral promotion properties, it would be advantageous to identify compounds which may be clinically relevant for opiate drug abusers. A previous study showing methadone maintenance restored the immunosuppressive profile of heroin abusers (Novick et al., 1989) has led one group to speculate methadone as a major preventive strategy for the spread of HIV-1 in

opiate drug abusers (McLachlan et al., 1993). Indeed, pharmacokinetics studies suggests methadone has no detrimental effects on selective parameters within the immune system in mice (Pacifci et al., 1994). Accordingly, the development of unique opioid compounds either peptides (Dooley et al., 1994) or alkaloid derivatives (Bagley et al., 1989; Zimmerman and Leander, 1990) with novel pharmacodynamics has provided a source by which pharmacologists and immunologists can initiate studies in order to identify those reagents that might hold promise in the clinical theater of applied science as it relates to AIDS and cancer.

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Mechanisms of Herpes Simplex Virus Type 1 Reactivation

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Primary cultures of trigeminal ganglion (TG) cells from herpes simplex virus type 1 (HSV-1) latently infected mice were used to study reactivation. Expression of HSV-1 latency-associated transcripts was noted in TG cell cultures. Infectious virus appeared in 75% of culture supernatants within 120 h after heat stress. Likewise, HSV-1 lytic-phase mRNA and proteins were detectable 24 h after heat stress. HSV-1 antigen first appeared in neurons after heat stress, indicating the neurons were the source of reactivation. The effect of heat stress duration on reactivation was determined. Reactivation occurred in 0, 40, or 67% of cultures after a 1-, 2-, or 3-h heat stress, respectively. However, 72-kDa heat shock protein expression was induced regardless of heat stress duration. Thus, reactivation was not a direct result of inducing the heat shock response. The capacities of several drugs to induce reactivation were also evaluated. While neither epinephrine, forskolin, nor a membrane-permeable cyclic AMP analog induced reactivation, dexamethasone did so in a dose-dependent manner. Furthermore, dexamethasone pretreatment enhanced the kinetics of heat stress-induced reactivation from TG cells. Collectively, the results indicate that TG cell cultures mimic important aspects of in vivo latency and reactivation. Therefore, this model may be useful for studying signalling pathways that lead to HSV-1 reactivation.

Despite two decades of research since sensory neurons were identified as the site of latent herpes simplex virus type 1 (HSV-1) infection (6), little is known about the mechanisms by which stress induces reactivation. While our knowledge of the HSV-1 genome and its gene products has increased tremendously, no new clinical treatments have been developed to block reactivation. Stressors such as epinephrine iontophoresis (19), cyclophosphamide and dexamethasone (Dex) administration (7), UV irradiation (20), and transient hyperthermia (35) induce reactivation in animal models. In no model, however, are the events that occur between stress and production of infectious HSV-1 known. Therefore, many unresolved questions remain. What are the reactivation-inducing signals that impinge upon latently infected neurons? Through what receptors are those signals recognized and transduced across the cell membrane? What intracellular effectors carry these signals to the nucleus? What changes in HSV-1 gene regulation constitute the event referred to as reactivation? Understanding these molecular mechanisms would greatly facilitate the rational development of reactivation-blocking drugs. Because such questions are difficult to address in vivo, an in vitro model which mimics the in vivo situation would provide an important tool for studying mechanisms of reactivation.

Culture systems in which HSV-1 reactivation occurs have been described, but most are poor models of in vivo latency and reactivation. For example, coculture of trigeminal ganglion (TG) explants with HSV-1 permissive cells is a useful assay for verifying latent infection (43) but is of limited value as a reactivation model because reactivation is induced regardless of treatment. Neonatal sensory neuron cultures are a good model for studying reactivation because HSV-1 latency is maintained until administration of a stressor (41, 48). How-

ever, this model differs substantially from adult ganglionic neurons in vivo. All of the cells in the neonatal cell cultures are neurons, and depending on the multiplicity of infection, up to 100% of these neurons can be latently infected. In contrast, 5 to 10% of cells in the ganglion are neurons, and only 5 to 10% of those become latently infected following HSV-1 ocular infection (14).

Moriya et al. (26) described a culture system which is potentially useful as an in vitro model of stress-induced reactivation. In the presence of an antiviral drug, primary cell cultures were established from latently infected mouse TGs. Latency was maintained following removal of the antiviral drug, and the investigators found that HSV-1 could be reactivated from TG cells by heat stress. Because critical points (e.g., demonstration that cytopathic effects were due to HSV-1) were not addressed in this study, the results were suggestive but not compelling.

In the present study, we confirm the results of Moriya et al. (26) and demonstrate that TG cell cultures mimic important aspects of in vivo latency and reactivation. Evidence is provided that (i) neurons remain viable in TG cell cultures, (ii) latency-associated transcription continues in culture, (iii) HSV-1 mRNA, protein, and virion synthesis are efficiently reactivated following heat stress, and (iv) latently infected neurons are the source from which HSV-1 reactivation occurs.

The capacities of cyclic AMP (cAMP), epinephrine, and Dex to induce HSV-1 reactivation from TG cell cultures were also studied. These compounds were chosen, in particular, because of their reactivation-inducing capacity in other herpesvirus models (19, 32, 41) and because of their relationship to the in vivo stress response. While epinephrine and cAMP had no effect on TG cells, Dex induced HSV-1 reactivation in a dose-dependent manner. Furthermore, Dex enhanced the kinetics of heat stress-induced reactivation. The results suggest a central role for glucocorticoid hormones in vivo in stress-induced HSV reactivation.

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TABLE 1. Oligonucleotide primers used in this study

Primer	Location in HSV-1	Sequence	Reference
G3PDH-a		GAATCTACTGGCGTCTTCACC	18
G3PDH-b		GTCATGAGCCCTTCCACGATGC	
ICP0-3'	←120987	TTCGACCAAGGGACCCCTAGT	
LAT-a	120702→	GACAGCAAAAATCCCCTGAG	22
LAT-b	←120896	ACGAGGGAAAACAATAAGGG	
ICP27-a	114922→	TTTCTCCAGTGTAGCTGAAGG	2
ICP27-b	←115204	TCAACTCGCAGACACGACTCG	
RR-a	88517→	ATGCCAGACCTGTTTTTCAA	15
RR-b	←88759	GTCTTTGAACATGACGAAGG	
TK-a	46622→	ATACCGACGATCTGCGACCT	22
TK-b	←46731	TTATTGCGCTCATAGCGCGG	

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MATERIALS AND METHODS

Animals and cells. Female 25- to 34-g ICR mice (Harlan-Sprague Dawley, Indianapolis, Ind.) were used in these experiments. The CV-1 African green monkey kidney cell line was obtained from the American Type Culture Collection (Rockville, Md.).

Infection of mice. Bilateral ocular infection of ICR mice (Harlan-Sprague Dawley) was performed as follows. Corneas were scarified with a 25-g needle, and tear film was blotted from the eyes with tissue. Mice were infected by placing 3 μ l of medium containing 10^5 PFU of HSV-1 (McKrae strain) per ml on each eye. At the time of infection, mice were passively immunized with 0.1 ml of rabbit antiserum to HSV-1 by intraperitoneal injection to enhance survival. To verify primary infection, mouse eyes were swabbed 2 days postinoculation, and swabs were transferred to CV-1 monolayer cultures, which were observed for HSV-1-induced cytopathic effects.

Establishment of TG cell cultures. TG cell cultures were prepared according to a modified version of the protocol described by Moriya et al. (26). TG cells were cultured in minimum essential medium containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, Md.), antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, Mo.), and 10 ng of nerve growth factor 2.5s (Collaborative Biomedical Products, Bedford, Mass.) per ml (TG medium). TGs were aseptically removed from latently infected mice (greater than 28 days postinoculation) and placed in TG medium on ice. TGs were pooled in 1.5 ml of calcium- and magnesium-free Hank's balanced saline solution containing collagenase type XI (1 mg/ml; Sigma) and collagenase type IV (1 mg/ml; Sigma) and incubated at 37°C for 75 to 90 min. To facilitate dissociation, ganglia were triturated every 25 min with a 1-ml serological pipette.

Next, 8.5 ml of TG medium was added to the dissociated cells, which were then pelleted by centrifugation (4°C, 5 min, 200 \times g), and the collagenase-containing supernatant was discarded. The cells were rinsed twice more with TG medium and resuspended in 50 ml of TG medium containing 5 μ g/ml of (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU; Sigma) per ml. The cells were distributed into two 24-well culture plates (1 ml of cell suspension per well) which had been thin coated with rat tail collagen type I (50 μ g/ml; Collaborative Biomedical Products) and recombinant mouse laminin (2.5 μ g/ml; Collaborative Biomedical Products) according to the vendor's directions. To promote cell adherence, culture plates were centrifuged for 3 min at 200 \times g.

Cultures were incubated in a 37°C tissue culture incubator (5% CO₂, 95% humidity). Two days after culture establishment, 0.5 ml of TG medium was added to each culture well. Five days after culture establishment, BVDU was removed by replacing the culture medium. Cultures were incubated for 14 to 16 days before initiation of reactivation experiments. Culture medium was partially replaced every 5 to 7 days.

Heat stress and drug treatments. TG cells were heat stressed by placing culture plates in a 43°C tissue culture incubator (5% CO₂, 95% humidity) for 3 h.

In drug experiments, cultures were incubated with either forskolin for 1 h, chlorophenylthio (CPT)-cAMP for 1.5 h, epinephrine for 1.5 h, or Dex for the duration of the experiment. Five days after drug treatment, cultures were heat stressed to verify the presence of latent HSV-1.

The effect of forskolin on intracellular cAMP concentrations was determined 30 min after addition of forskolin to TG cells, using a cAMP enzyme-linked immunosorbent assay (PerSeptive Diagnostics, Cambridge, Mass.).

Monitoring of TG cultures for HSV-1 reactivation. Infectious HSV-1 was detected in cultures by transferring 100 μ l of culture medium to CV-1 cell monolayers in 96-well plates and monitoring CV-1 cells for the appearance of cytopathic effect. Each TG culture well was sampled for infectious virus at 4, 8, and 12 days after plating and immediately before administration of a potential

reactivation-inducing stimulus. Thereafter, wells were sampled every 24 h for 5 days to record the appearance of infectious virus.

Reverse transcription-PCR (RT-PCR). Total RNA was extracted directly from culture wells with Ultraspec RNA isolation reagent (Biotecx Inc., Houston, Tex.) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on equivalent amounts of RNA from each sample, using an oligo(dT)₁₅ primer and avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, Wis.). Because the major species of latency-associated transcripts (LAT) is not polyadenylated (10), reverse transcription of LAT was achieved by using a LAT-specific primer, ICP0-3'. PCR was performed on equivalent amounts of cDNA; each reaction mixture contained 1 \times Taq buffer, 0.25 μ M each PCR primer, 100 μ M each deoxynucleoside triphosphate, and 2.5 U of Taq polymerase (Promega), and each reaction was performed in an MJ Research (Watertown, Mass.) thermal cycler with 35 cycles of 94°C (1 min 15 s) \rightarrow 57°C (1 min 15 s) \rightarrow 72°C (30 s). Oligonucleotide primers were obtained from LSU Medical Center Core Laboratories (New Orleans, La.) and are listed in Table 1. Densitometry of ethidium bromide-stained agarose gels was performed with an EagleEye II still video system (Stratagene, La Jolla, Calif.).

Immunocytochemical staining. Cell cultures were fixed with 10% formalin and treated for 10 min with 0.1 N sodium azide containing 0.3% H₂O₂ to inactivate endogenous peroxidases. HSV-1 antigen was identified by using a rabbit anti-HSV-1-horseradish peroxidase conjugate (DAKO Corporation, Carpinteria, Calif.). Neurons were labeled with rabbit anti-neuron-specific enolase (Zymed Laboratories Inc., San Francisco, Calif.), and the primary antibody was detected with a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, Calif.). Antibody labeling was visualized with the substrate aminoethylcarbazole (Vector Laboratories).

Western blot (immunoblot) analysis. Following lysis of cultured cells in non-denaturing gel loading buffer, debris was pelleted by centrifugation. Aliquots of clarified supernatant were loaded on sodium dodecyl sulfate-7.5% polyacrylamide minigels. Separated proteins were electroblotted onto nitrocellulose (BioBlot NC; Corning Costar, Cambridge, Mass.) and fixed in methanol-Tris buffer. A mouse anti-72/73-kDa heat shock protein monoclonal antibody (Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada) was used to detect 72-kDa heat shock protein HSP-72, which was visualized with the Western Light chemiluminescence detection system (Tropix Inc., Bedford, Mass.).

RESULTS

Neurons in TG cell cultures. Nerve fibers are the conduit by which HSV-1 enters the ganglion during acute infection, and neurons are the only known reservoir of viral DNA during latency (24, 30, 45). Therefore, we sought to verify that neurons survive ganglion dissociation and remain viable in TG cell cultures. Neurons were identified by light microscopy (Fig. 1A) on the basis of their large size and distinct nucleus. Immunocytochemical staining for neuron-specific enolase showed that these cells were in fact neurons (Fig. 1B). The number of neuron-specific enolase-positive cells per culture well was determined to be 410 ± 48 (mean \pm standard deviation; $n = 5$ wells). Because each well received 0.4 ganglion equivalent of cells, approximately 1,000 neurons per ganglion survived dissociation and became adherent in culture.

Latency-associated transcription. During latent infection of the TG in vivo, HSV-1 LATs are detected exclusively in latently infected neurons by in situ hybridization (42, 44). Because TGs were taken from latently infected mice (greater than 28 days postinoculation), RT-PCR analysis was performed to determine if LAT expression persisted in TG cells following dissociation. LATs were detected in 10 of 10 cultures 14 days after establishment (Fig. 2). Presumably, latently infected neurons which survived dissociation are the source of LAT expression in TG cell cultures. However, in situ hybridization will be required to formally address this point.

Effect of BVDU in establishment of TG cell cultures. TG cells were initially cultured in the presence of an antiviral drug, BVDU (9), to prevent reactivation following ganglion dissociation. Contrary to previous results (26), reactivation did not always occur from TG cells established in the absence of antiviral drug. Reactivation occurred in 57% (16 of 28) of cultures established in the absence of BVDU. The lack of reactivation from the other TG cells was not due to an absence of

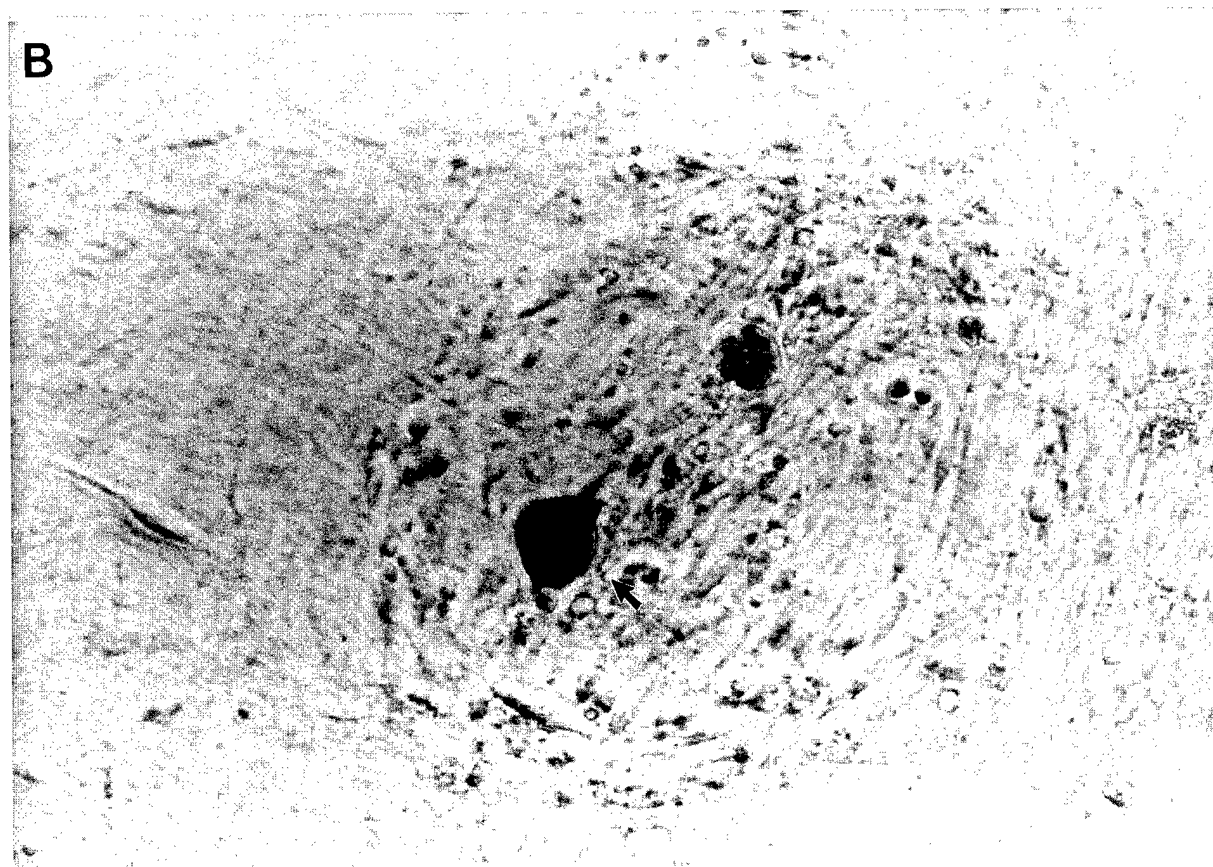
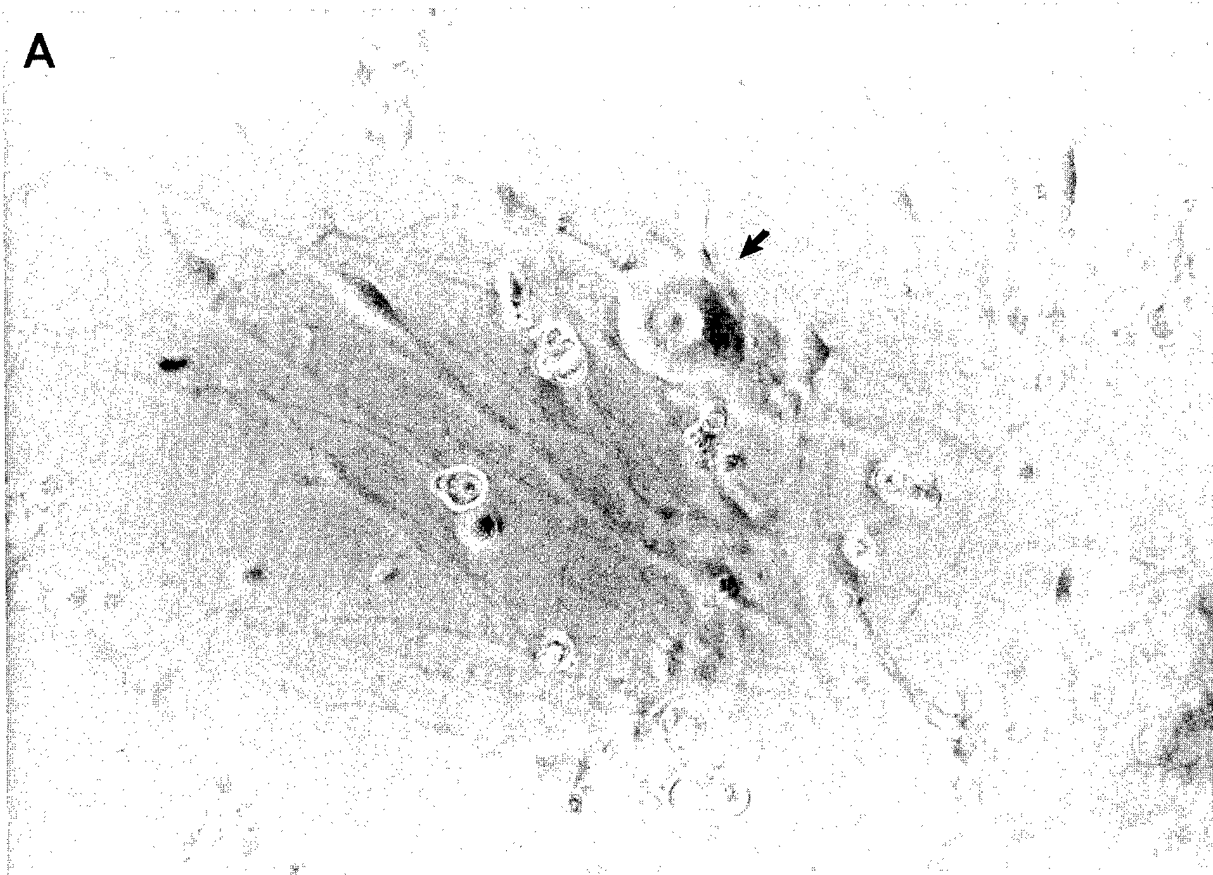


FIG. 1. Neurons in TG cell cultures. (A) Phase-contrast appearance of a neuron in culture (indicated by arrow) (magnification, $\times 40$). Note regenerated neurite extending from left side of neuron. (B) Immunocytochemical staining reveals the presence of neuron-specific enolase (indicated by arrow) (magnification, $\times 20$).



FIG. 2. Agarose gel analysis of LAT RT-PCR products (195 bp) amplified from cell cultures 1 to 10 (lanes 1 to 10). Each RNA sample was derived from the cells in a single culture well. No template (-) and HSV-1 DNA (+) served as negative and positive controls, respectively, in the PCR assay. ϕ X174/*Hae*III markers are shown on the far left.

latent HSV-1; reactivation later occurred from 10 of the 12 remaining cultures following heat stress.

To determine the drug concentration which would consistently prevent HSV-1 reactivation, cultures were established in medium containing half-log dilutions of BVDU ranging from 0.5 to 50 μ g/ml. Reactivation did not occur in cultures containing 1.6 μ g or more of BVDU per ml ($n = 4$ per group).

RT-PCR analysis of HSV-1 gene expression was performed to verify the efficacy of 5 μ g of BVDU per ml in preventing HSV-1 reactivation during culture establishment. Infected cell polypeptide 27 (ICP27) mRNA was present 24 h after culture establishment but was not detectable by 120 h (Fig. 3A). Therefore, HSV-1 immediate-early gene expression is only transiently upregulated in BVDU-treated cultures. In contrast, expression of HSV-1 ICP27 mRNA increased steadily after culture establishment in the absence of BVDU (Fig. 3A). While LAT expression was detected at all times in BVDU-treated cultures, LAT expression was downregulated in 3 of 4 untreated cultures during the 24- to 48-h time period when reactivation was first occurring (Fig. 3A). PCR of TG cell culture DNA verified that 5 μ g of BVDU per ml effectively prevents HSV-1 DNA replication following culture establish-

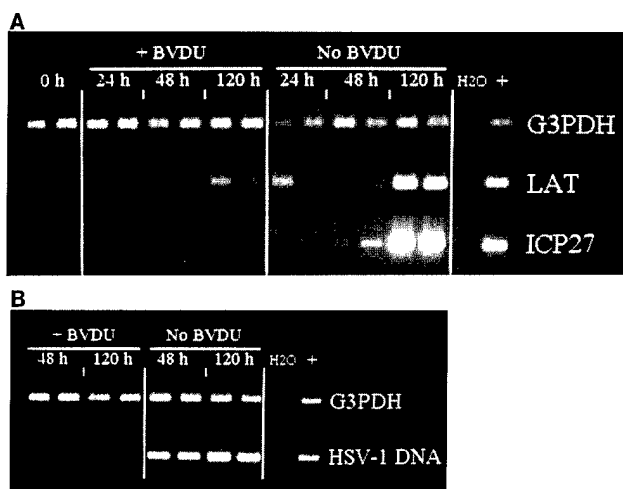


FIG. 3. BVDU treatment prevents HSV-1 reactivation. (A) Effect of BVDU on HSV-1 immediate-early gene expression, determined by RT-PCR analysis of RNA from duplicate samples of TG cells taken 0, 24, 48, and 120 h after establishment of TG cell cultures. The cells were all derived from the same pool of dissociated TGs. + BVDU, cultures that were BVDU treated; No BVDU, cultures that were untreated. RT-PCR was used to detect G3PDH, LAT, and ICP27 mRNAs. Each RNA sample was derived from the pooled cells of three culture wells. (B) Effect of BVDU on HSV-1 DNA replication. PCR was performed on equivalent amounts of DNA from TG cell cultures 48 and 120 h after culture establishment to detect the cellular G3PDH gene and HSV-1 DNA. HSV-1 ribonucleotide reductase primers were used to detect HSV-1 DNA. Each DNA sample was derived from the pooled cells of three culture wells. No template (H₂O) and HSV-1 DNA (+) served as negative and positive controls, respectively, in each PCR assay.

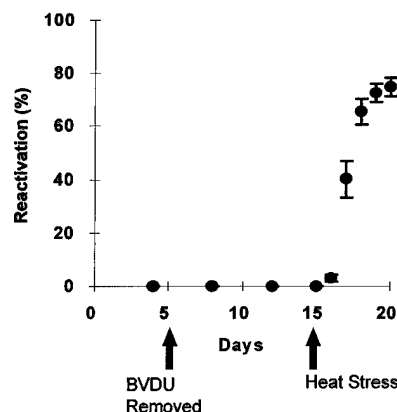


FIG. 4. Kinetics of heat stress-induced HSV-1 reactivation. Heat stress was administered 15 days after culture establishment. Reactivation is defined as the appearance of infectious virus in culture supernatants. Datum points represent the mean frequencies of reactivation (\pm standard errors of the means) observed in 11 experiments.

ment. Whereas amplification of HSV-1 DNA in untreated cultures was evident by 48 and 120 h after culture establishment, HSV-1 DNA levels remained constant in BVDU-treated cultures (Fig. 3B).

Heat stress-induced HSV-1 reactivation. Reactivation did not occur in any cultures following removal of BVDU from cells (5 days after culture establishment). However, heat stress rapidly induced HSV-1 reactivation (Fig. 4) from $74.7\% \pm 3.5\%$ of TG cell cultures ($n = 257$ wells). This finding is consistent with the results of Moriya et al. (26). Of the cultures which eventually reactivated, HSV-1 was detectable in 88% of culture supernatants within 72 h after heat stress. In contrast, reactivation occurred from 0% of nonstressed controls ($n = 52$) over a 20-day culture period.

Heat stress induction of HSV-1 gene expression. In most experiments, reactivation was defined as the appearance of infectious virus in culture supernatants which caused cytopathic effect when virus was transferred to CV-1 cell monolayers. To confirm that heat stress triggered HSV-1 reactivation from TG cells, heat stress induction of ICP27 mRNA expression was verified by RT-PCR (Fig. 5). While ICP27 mRNA was not detectable before or 12 h after the initiation of heat stress, ICP27 mRNA was evident in TG cell cultures by 24 and 48 h after heat stress. In contrast, glyceraldehyde-3-phosphodehydrogenase (G3PDH) mRNA was uniformly present in all RNA samples. After normalization for G3PDH, densitometry indicated that the amount of ICP27 PCR product amplified from TG cell RNA samples increased 18-fold between 24 and 48 h after heat stress.

Immunocytochemical staining was used to determine the kinetics of HSV-1 protein induction following heat stress. Cultured cells did not express detectable HSV-1 protein prior to

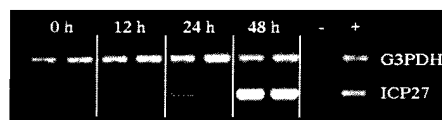


FIG. 5. Kinetics of ICP27 mRNA induction following heat stress. RT-PCR was used to detect G3PDH and HSV-1 ICP27 mRNAs in duplicate samples of TG cells prior to heat stress (0 h) and at 12, 24, and 48 h after initiation of heat stress. No template (-) and HSV-1 DNA (+) served as negative and positive controls, respectively, in each PCR assay. The samples shown are a subset of VEH PCR samples shown in Fig. 10.

heat stress. However, HSV-1 proteins were detected in TG cell cultures 22, 48, and 72 h after heat stress (Fig. 6A to C). 1HSV-1 antigen expression was limited to large, neuron-like cells 22 h after heat stress (Fig. 6D). Large foci of HSV-1 antigen-positive cells were seen 48 and 72 h after heat stress. On the basis of the discrete, circular pattern of staining, each HSV-1 antigen-positive cell cluster appeared to be the result of focal spread from a single reactivation event. The average numbers of reactivation events detected per culture well were 0.5 ($n = 8$ wells), 1.5 ($n = 4$ wells), and 1.75 ($n = 4$ wells) at 22, 48, and 72 h after heat stress, respectively. Therefore, it appears that only a fraction of neurons in which reactivation occurred contained detectable HSV-1 protein by 22 h after heat stress.

Effect of heat stress duration on HSV-1 reactivation. The effect of heat stress duration on TG cell cultures was studied to better characterize the mechanism by which heat stress induces HSV-1 reactivation. TG cells were heat stressed for 1, 2, and 3 h at 43°C and observed for HSV-1 reactivation (Fig. 7, 1st heat stress). Consistent with other experiments, HSV-1 reactivated from 67% (8 of 12) of TG cell cultures heat stressed for 3 h. However, 2 h of heat stress induced reactivation from only 40% of cultures ($n = 28$), and 0% of cultures ($n = 28$) reactivated following 1 h at 43°C. To verify the presence of reactivatable virus, the cultures previously treated for 1, 2, or 3 h were heat stressed a second time for 3 h (Fig. 7, 2nd heat stress). After a 3-h heat stress, HSV-1 reactivated from 61% of cultures previously heat stressed for 1 h. Likewise, the frequency of reactivation increased from 40 to 61% in those cultures previously heat stressed for 2 h. Interestingly, the frequency of reactivation increased from 67 to 92% in cultures that had been heat stressed for 3 h the first time. Twelve TG cell cultures that were not heat treated did not reactivate over the 25-day course of this experiment.

The results of others suggest that HSV-1 reactivation may be triggered by induction of the cellular heat shock response (26, 35). Expression of HSP-72 (often used as a marker of heat shock) was rapidly induced in TG cells following transfer to a 43°C incubator. While HSP-72 was not detected before or 1 h after transfer to 43°C, HSP-72 expression was evident 2 and 3 h after initiation of heat stress (Fig. 8A).

To account for the observed lack of reactivation after 1 h of heat stress, we thought that perhaps 1 h in a 43°C incubator was insufficient to induce a heat shock response. Therefore, the effect of heat stress duration on HSP-72 induction was determined as follows. Cultures were simultaneously placed at 43°C. One culture was returned to 37°C after 1 h, one culture was returned to 37°C after 2 h, and the other culture remained at 43°C for the entire 3 h. Protein samples were harvested from all cultures 3 h after initiation of heat stress, and levels of HSP-72 expression were compared by Western blot analysis. HSP-72 expression was induced to high levels in TG cells regardless of heat stress duration (Fig. 8B). Therefore, HSV-1 reactivation in TG cells is not a direct result of triggering a heat shock response.

Analysis of potential reactivation-inducing drugs. It has been reported that LAT facilitates reactivation (16), and cAMP has been proposed to contribute to reactivation by upregulating LAT transcription (21, 29). Smith et al. (41) found that either 500 μ M CPT-cAMP or 50 μ M forskolin (i.e., an activator of adenylyl cyclase) induced reactivation from neonatal neuron cultures. Likewise, epinephrine and glucocorticoids (e.g., Dex) have been used in animal models to induce reactivation of latent herpesvirus (19, 32, 46). Therefore, the reactivation-inducing potentials of cAMP, epinephrine, and Dex were studied in TG cell cultures to compare this system with other reactivation models.

While 0.5 μ M forskolin did not elevate cAMP above basal levels (1.4 ± 1.7 pmol of cAMP per mg of protein [mean \pm standard deviation]), cAMP levels were nearly 10-fold higher in TG cells 30 min after treatment with 50 μ M forskolin (12 ± 2.6 pmol of cAMP per mg of protein; mean \pm standard deviation). However, neither 50 μ M forskolin nor 500 μ M CPT-cAMP induced reactivation in TG cell cultures (Fig. 9). Epinephrine also had no effect in TG cell cultures. However, Dex induced reactivation in TG cells in a dose-dependent manner (Fig. 9). Following the 5-day period allowed for drug-induced reactivation, cultures were heat stressed to verify the presence of latent virus. Heat stress confirmed that in each treatment group, the majority of TG cell cultures contained reactivatable HSV-1 (i.e., 60 to 90% of cultures reactivated within 5 days after heat stress). As a control, some of the drug-treated cultures were not secondarily heat stressed; reactivation did not occur in these cultures.

Following heat stress, HSV-1 was detectable in culture supernatants of Dex-pretreated cells earlier than untreated controls. Likewise, comparison with results of previous experiments indicated that HSV-1 appeared significantly earlier in Dex-pretreated cultures (Table 2). Because of the known role of the glucocorticoid receptor as a transcriptional regulator (13, 31, 36), we postulated that Dex facilitated the induction of HSV-1 lytic-phase mRNA transcription following heat stress.

RT-PCR analysis of Dex- and vehicle-treated TG cells substantiated this hypothesis (Fig. 10). While viral mRNA was barely detectable in one of two vehicle-treated cultures 12 h after heat stress, viral transcripts for ICP27, thymidine kinase (TK), and ribonucleotide reductase (RR) were evident in two of two Dex-pretreated cultures 12 h after heat stress. Furthermore, detection of ICP27, TK, and RR mRNAs indicated that reactivation occurred in one of two DEX pretreated cultures that was not heat stressed. Because Dex was added just 15 h prior to extraction of RNA, the brief interval between stimulus and response suggests that Dex acted directly through neuronal glucocorticoid receptors to induce reactivation.

Despite the presence of ICP27, TK, and RR mRNAs, LAT was not detected in the Dex-treated culture that reactivated without being heat stressed. Interestingly, LAT was also not detected in vehicle-treated cultures 24 h after heat stress, despite the presence of lytic phase transcripts. On the basis of previous immunocytochemical staining experiments, RT-PCR was presumably detecting viral transcription in just one or two reactivation-positive cells. Therefore, the absence of LAT in these samples suggests a coordinated downregulation of LAT transcription during reactivation. Such an event has been proposed by Rock et al. (32) and could be mediated by ICP4, given the capacity of this HSV-1 regulatory protein to repress LAT transcription (1).

DISCUSSION

Establishment of TG cell cultures. The explantation of TG cells provides a reactivation-inducing stimulus to latently infected neurons during culture establishment. This is demonstrated by the upregulation of ICP27 mRNA expression in BVDU-treated and untreated cultures 24 to 48 h after culture establishment. Because the TG cells are plated as a single-cell suspension, however, the antiviral drug is effective immediately. Therefore, despite the induction of immediate-early gene expression, BVDU blocks HSV-1 DNA replication and prevents infectious virus production during culture establishment. The absence of ICP27 mRNA in BVDU-treated cells by 120 h after culture establishment indicates that the reactivation-inducing stimulus associated with explantation is tran-

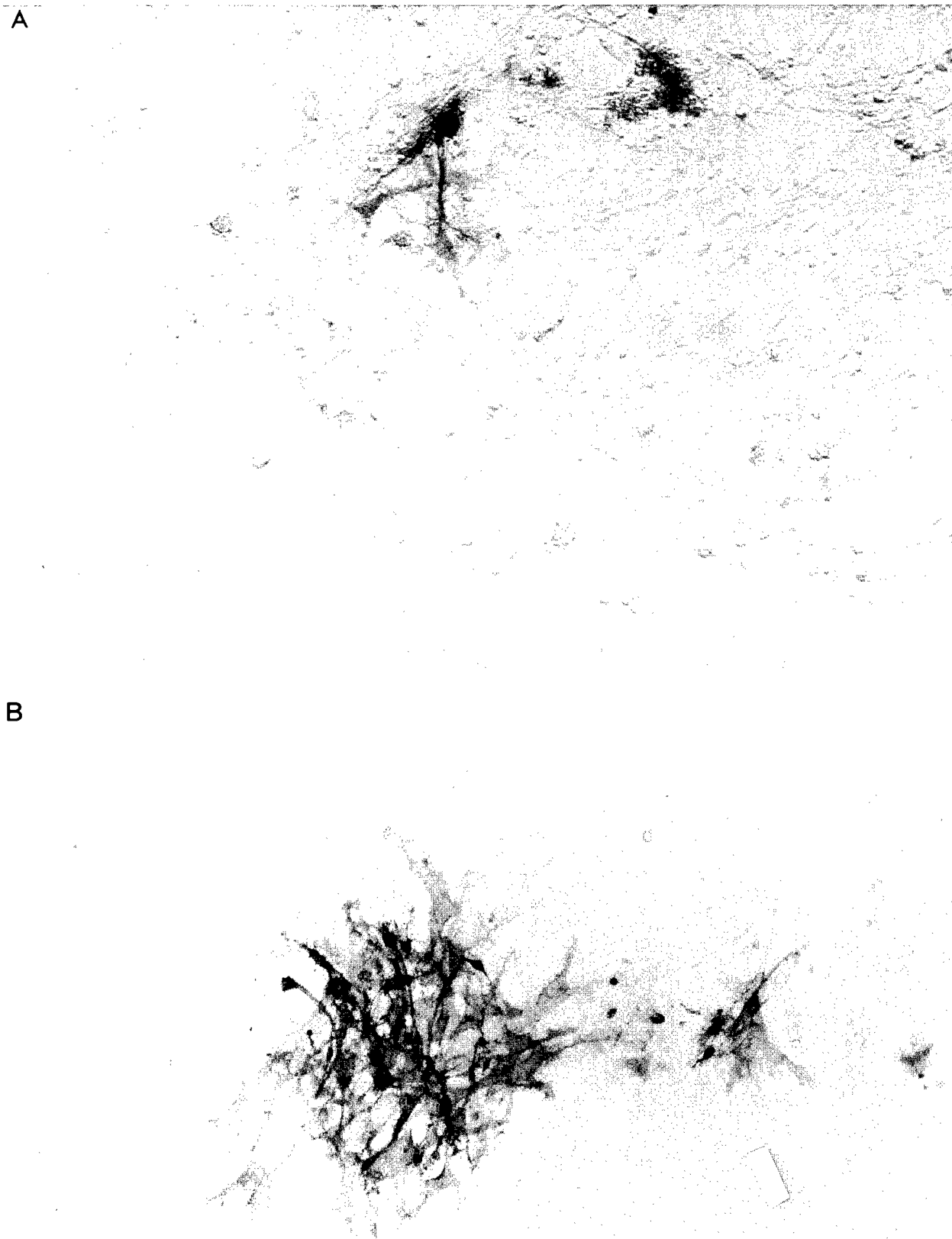


FIG. 6. Kinetics of HSV-1 protein induction following heat stress. TG cell cultures stained for HSV-1 antigen 22 h (A), 48 h (B), and 72 h (C) after initiation of heat stress (magnification, $\times 10$). (D) A neuron stained for HSV-1 antigen 22 h after heat stress (magnification, $\times 40$). All HSV-1 antigen-positive cells identified at this time point had a large nucleus and a prominent nucleolus, characteristic of neurons.

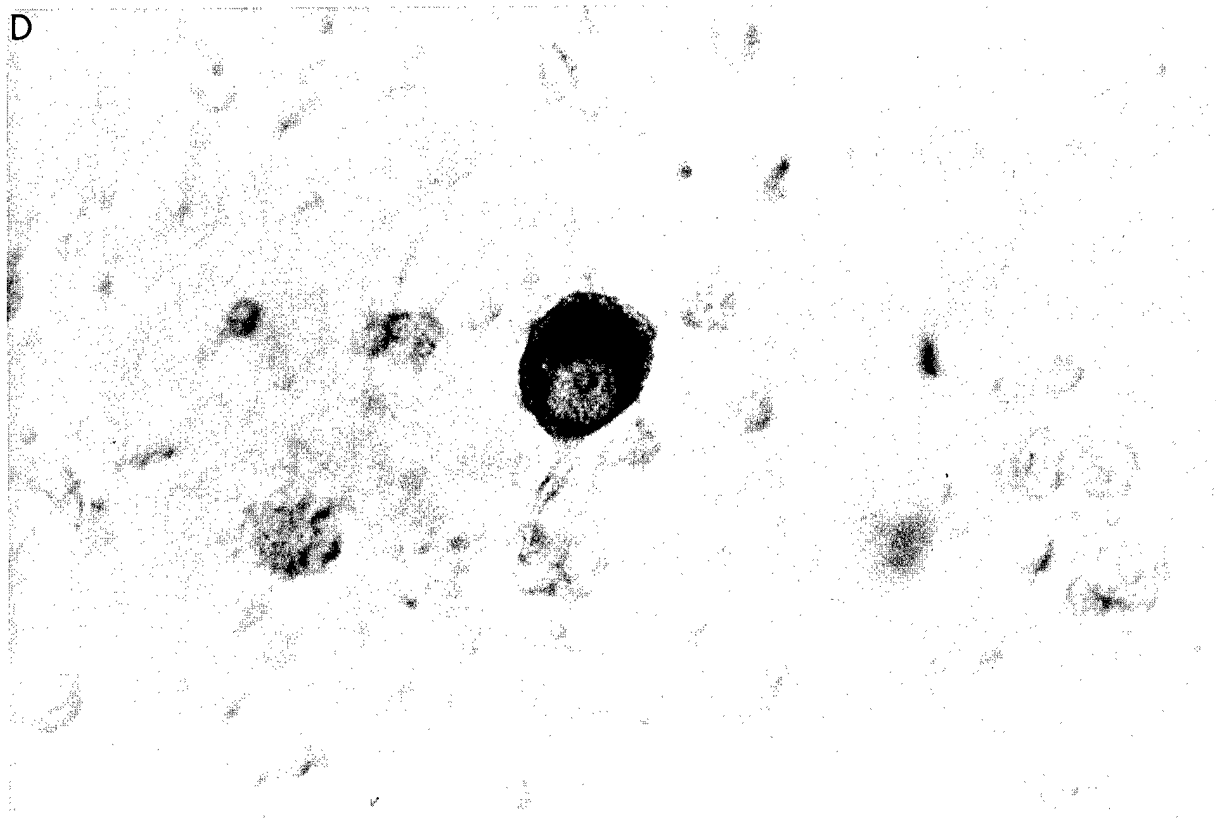
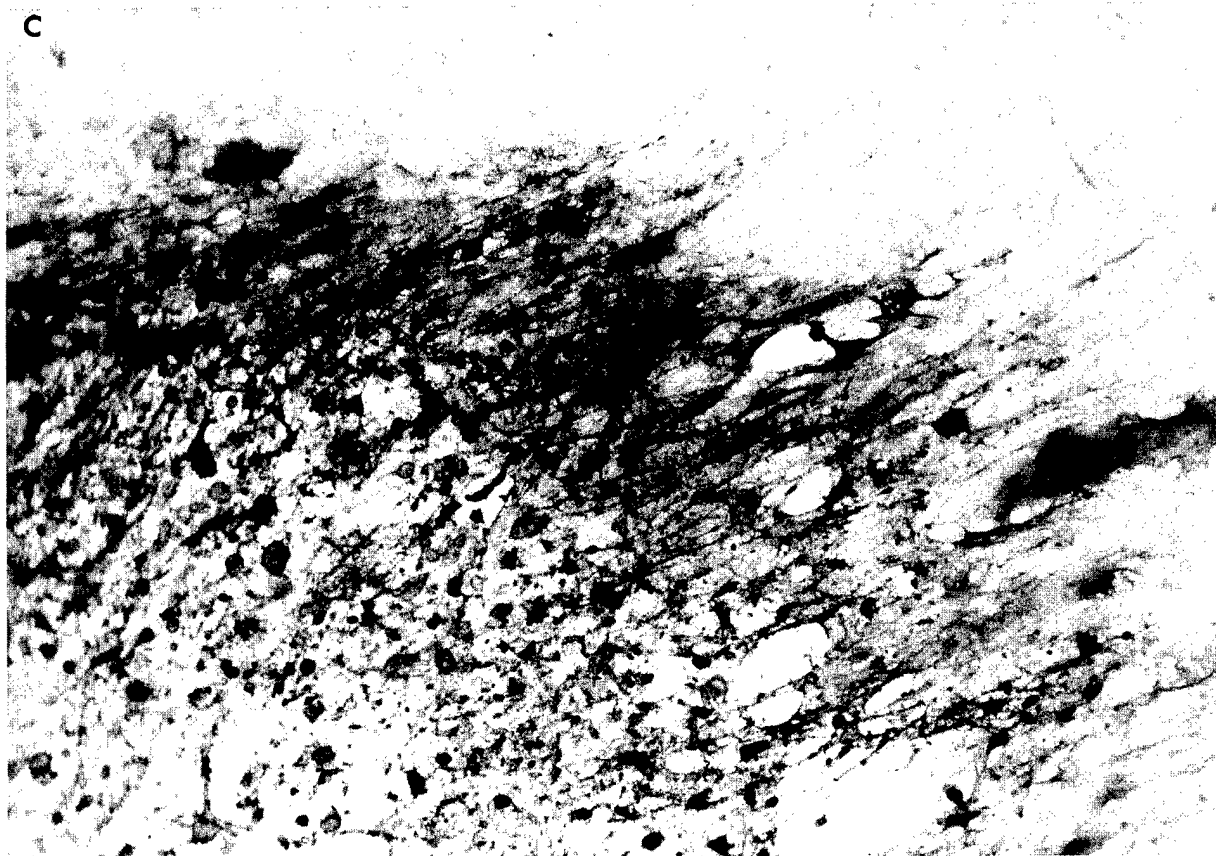


FIG. 6—Continued.

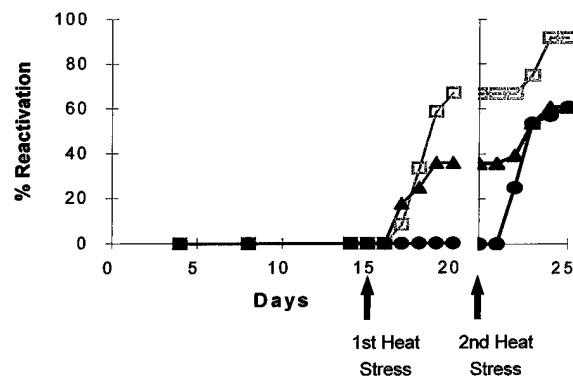


FIG. 7. Effect of heat stress duration on HSV-1 reactivation. Shown are percent reactivation following a 1-h (●), 2-h (▲), or 3-h (□) heat stress administered after 15 days in culture and percent reactivation following a second 3-h heat stress after 20 days in culture.

sient. Consistent with this hypothesis, throughout these studies reactivation was not observed following BVDU removal from TG cell cultures.

Reactivation from TG cells. In animal models, HSV-1 mRNA and antigen production in the ganglion is transient during stress-induced reactivation (2, 35). In light of evidence that T lymphocytes and cytokine expression persist in the TG after latency is established (4, 11, 40), the brevity of HSV-1 reactivation in vivo presumably reflects an efficient host immune response. In contrast, induction of reactivation in TG cell cultures is irreversible because lytic replication continues until all cells are destroyed.

Although HSV-1 remained largely undetected in culture medium until 48 h after heat stress, replication had initiated in neurons by 24 h after heat stress. The frequency of detecting HSV-1 in culture supernatants increased from 3 to 40% between 24 and 48 h after heat stress. HSV-1 antigen expression was restricted to neurons 22 h after heat stress but spread to plaque-like clusters of 50 to 500 cells by 48 h after heat stress. Likewise, RT-PCR indicated that the amount of ICP27 mRNA in TG cells increased substantially between 24 and 48 h after heat stress. Therefore, HSV-1 was not usually detected in culture medium until released from secondarily infected cells.

None of the treatments were 100% effective in inducing reactivation. In testing the effects of heat stress duration, 8 of 12 cultures reactivated after a first 3-h heat stress interval, but 3 of the remaining 4 cultures reactivated after a second 3-h heat stress. Likewise, while reactivation occurred in 16 of 28 cultures established in the absence of BVDU, 10 of the remaining 12 cultures reactivated following a secondary heat stress. Combined with the detection of LATs in 10 of 10 cultures, the results suggest that when cells are stressed, reactivation is initiated in only a fraction of latently infected neurons.

Mechanisms of HSV-1 reactivation. (i) Heat stress. While

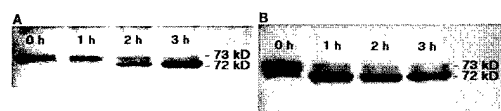


FIG. 8. Heat shock protein induction. (A) Kinetics of HSP-72 induction in TG cells 1, 2, and 3 h after transfer to a 43°C incubator (20 µg of protein per lane). (B) Effect of heat stress duration (1, 2, or 3 h) on heat shock protein induction by 3 h after transfer to 43°C incubator (35 µg of protein per lane).

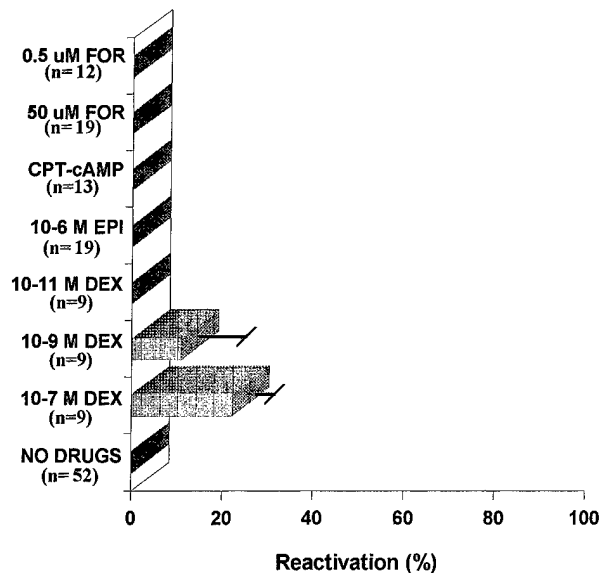


FIG. 9. Effects of drugs on reactivation. Frequency of drug-induced reactivation (\pm standard error of the mean) in TG cells treated with forskolin (FOR), CPT-cAMP, epinephrine (EPI), and Dex. Cultures were treated with drugs after 15 days in culture and observed for 120 h for reactivation.

HSP-72 expression was strongly induced in TG cells by 1 h of heat stress, reactivation occurred in 0 of 28 TG cell cultures incubated at 43°C for 1 h. Therefore, reactivation of HSV-1 gene expression is not a direct consequence of activating the signalling pathways which upregulate HSP-72 expression. However, heat stress induces HSP-72 expression more readily in glial cells than in neurons (34). Therefore, we cannot formally rule out the possibility that 1 h at 43°C induced HSP-72 expression in glial cells but was insufficient to activate the heat shock response in latently infected neurons.

(ii) Drugs. Stress stimulates epinephrine release from the sympathetic nervous system and glucocorticoid release from the adrenal glands. Given their roles as stress mediators, we hypothesized that these compounds serve as reactivation-inducing signals which act directly on latently infected neurons (i.e., ligand binding to neuronal adrenergic receptors or glucocorticoid receptors). In the case of epinephrine, this view is consistent with the finding that propranolol (β -adrenoceptor

TABLE 2. Enhancement by Dex of the rate of HSV-1 appearance following heat stress

Group	% reactivation ^a at indicated time poststress (mean \pm SEM)		
	24 h	48 h	72 h
10 ⁻⁷ M Dex ^b	26 \pm 11 ^c	50 \pm 17	100 \pm 0
10 ⁻⁹ M Dex ^b	0 \pm 0	90 \pm 10 ^c	100 \pm 0
10 ⁻¹¹ M Dex ^b	6 \pm 6	89 \pm 7 ^c	100 \pm 0
No Dex ^b	8 \pm 8	48 \pm 11	88 \pm 13
Expected ^d	2 \pm 4	46 \pm 13	84 \pm 6

^a Considering only those cultures that eventually reactivated, results represent percentages of reactivated wells at each time point after heat stress.

^b $n = 4$ experiments; four to five wells per experiment.

^c $P < 0.05$ (determined by analysis of variance and Tukey's post comparing Dex treatment groups with expected).

^d Pooled results of all other heat stress experiments ($n = 7$ experiments; 20 to 36 wells per experiment).

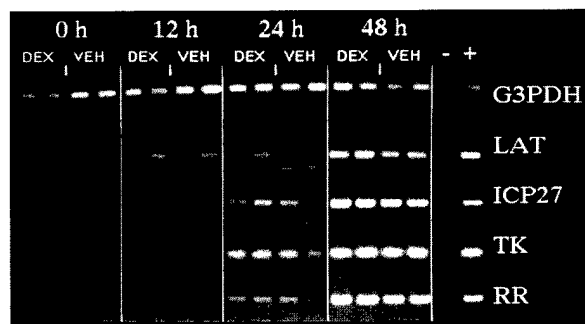


FIG. 10. Dex pretreatment enhances the rate of appearance of HSV-1 lytic phase mRNA following heat stress. Shown is RT-PCR analysis of RNA from duplicate samples of TG cells prior to heat stress (0 h) and at 12, 24, and 48 h after initiation of heat stress. Dex, cultures which were treated with 10^{-7} M Dex 15 h before heat stress; VEH, cultures which were vehicle treated 15 h before heat stress. RT-PCR was used to detect G3PDH, LAT, ICP27, HSV-1 TK, and HSV-1 RR mRNAs. Each RNA sample was derived from the pooled cells of two culture wells. No template (-) and HSV-1 DNA (+) served as negative and positive controls, respectively, in each PCR assay.

antagonist) blocks reactivation from mice following hyperthermic stress (12).

Epinephrine did not induce reactivation of latent HSV-1 in TG cell cultures. Therefore, ocular iontophoresis of epinephrine may not act directly on neurons to induce HSV-1 reactivation in vivo. Rather, epinephrine iontophoresis may stimulate corneal cells, which in turn transmit a reactivation signal to the nerve endings of the ganglionic neurons. Denervation of the cornea has been shown to block epinephrine-induced reactivation of HSV-1 (33). Therefore, we cannot rule out the possibility that epinephrine fails to induce reactivation in vitro because the regenerated neurites of the neurons lack an essential component that is present in vivo. In contrast, because Dex and cAMP act on sites in the neuronal cell body, these results are more pertinent to the in vivo situation. Therefore, the inability of forskolin and CPT-cAMP to induce reactivation suggests that cAMP elevation is not a critical determinant of HSV-1 reactivation in ganglionic neurons.

Dex treatment induced HSV-1 reactivation in a fraction of TG cell cultures. Underwood and Weed (46) likewise found that glucocorticoids (i.e., prednisone) induced reactivation from a low but significant fraction (17%) of HSV-1 latently infected mice. Given these observations, one could hypothesize that while glucocorticoid receptors facilitate HSV-1 reactivation, other unidentified signals are necessary for efficient induction. Therefore, Dex may serve as more than an immunosuppressant when used in concert with cyclophosphamide and UV radiation to induce HSV-1 reactivation in animal models (7, 39). In contrast, Dex induces rapid reactivation of bovine herpesvirus 1 in nearly 100% of latently infected animals (8, 28, 32, 38, 47) and induces efficient reactivation of pseudorabies virus in latently infected swine (25).

There is clinical precedence for herpes simplex and herpes zoster reactivation following systemic corticosteroid treatment (3, 5, 17, 23, 27, 37). The results of the present study suggest that glucocorticoids may contribute to disseminated herpetic disease not only by suppressing immune function but also by providing a reactivation trigger to latently infected ganglion.

Application of the in vitro reactivation model. Because dissociated TG cell cultures mimic key aspects of in vivo latency and reactivation, this paradigm offers a valuable tool for studying aspects of reactivation that are not readily addressed in vivo. For example, antisense inhibition could be used in cultures to identify viral and cellular genes that modulate reactivation.

Likewise, reagents whose use is prohibitively expensive in vivo (e.g., recombinant proteins and monoclonal antibodies) can be used cost-effectively in vitro. Because the method used to detect reactivation does not require harvesting the cells, TG cell cultures are available for secondary manipulations after testing of potential reactivation-inducing stimuli. In contrast, detection of reactivation in vivo often requires sacrificing the host. Finally, TG cell cultures provide a means to identify treatments which act directly on ganglion cells to induce or block reactivation.

Inevitably with cell culture studies, one is left with the question, "How does this pertain to the in vivo situation?" At least with primary cell cultures, only the milieu is changed. Presumably in TG cultures, reactivation is induced from the same cells in which reactivation would occur in vivo. Therefore, we believe that this model can be used to identify molecular events associated with reactivation and generate hypotheses whose relevance can be determined in vivo. In this context, while the results of this study suggest a central role for glucocorticoid receptors in induction of HSV reactivation, additional in vivo studies are required to substantiate this hypothesis.

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THE RELEVANCE OF OPIOIDS AND OPIOID RECEPTORS ON IMMUNOCOMPETENCE
AND IMMUNE HOMEOSTASIS

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Introduction

Since the previous review on the role of opioids in the immune system (1), numerous investigative teams have contributed to the growing pool of information illustrating the tangible relationship between opioids and immune function particularly as this association pertains to bacterial and viral pathogens. In addition, the recent cloning of both neural- and immune-derived opioid receptors will ultimately facilitate the identification of molecular events that are responsible for the immunomodulatory effects that are mediated by receptor ligation. Specifically, the administration of opioids *in vivo* can potentially affect the immune system either through direct interaction with receptors on the effector cells or indirectly, through the ligation of receptors found within the central nervous system. This indirect routing is hypothesized to involve secondary pathways including the hypothalamic pituitary adrenal (HPA) axis and the sympathetic nervous system ultimately resulting in immunomodulation (2). Consequently, a portion of this review will address the recent data on leukocyte-derived opioid receptors and the potential immunoregulatory role relative to opioid receptors found within the central nervous system. In addition, recent observations on the effects of opioids and immunocompetence will be reviewed from both a molecular and cellular perspective. Finally, the consequence of opioid exposure on the competence of the host immune system to microbial pathogens will be summarized.

Direct and indirect effects of opioids on immune function

Evidence for the existence of opioid receptor expression on cells of the immune system stemmed from the observations showing leukocytes could respond specifically to opioid ligands (3). Since these initial observations, the significance of opioid receptor expression on leukocytes has been under intense investigation. The direct effects of opioids on cells of the immune system have most recently been studied measuring macrophage activation and antibody production. Specifically, the prototypic μ agonist morphine and μ -selective agonist [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin (DAMGO), κ -selective agonist U50,488, and δ -selective agonist [D-Pen^{2,5}]enkephalin (DPDPE) have been shown to suppress peritoneal phagocytosis of *Candida albicans* through selective opioid antagonist-reversible mechanisms (4,5). Granulocyte chemotaxis or phagocytosis has also been shown to be suppressed in the presence of morphine (6, 7). Conversely, other results show that the endogenous opioid peptides dynorphin A, [met]- and [leu]-enkephalin, and β -endorphin enhance macrophage activation measuring tumoricidal activity, and the augmentation is antagonized by naloxone (8). Since the parameters measured are distinct, it is difficult to speculate as to why the discrepancies exist.

Opioids have also been shown to suppress antibody production. The original observations showing that κ agonists (U50,488 and U69,593) inhibited antibody production *in vitro* (9) could be mimicked by pretreating either T lymphocytes or macrophages with U50,488 prior to the *in vitro* plaque forming assays (10). The inhibition could be blocked with naloxone suggesting the direct effect of the agonist on the lymphocyte or macrophage. Similar to the *in vitro* effects of κ agonists, the administration of the κ agonist MR2034 *in vivo* has been shown to suppress the antibody response to sheep red blood cells *in vitro* (11, 12). Moreover, this effect was antagonized by peripheral administration of quaternary naltrexone, a compound that does not readily cross the blood-brain barrier (13). The effects of opioids on humoral immunity may be related to the modification to cytokine specific synthesis by T cells

and macrophages following stimulation (14-17). The T-dependent nature of opioid-induced suppression of humoral immunity is supported by data showing the active stereoisomer of morphine ([-]-morphine) has no effect on the humoral immune response to the T-independent antigen TNP-ficoll (18). Similarly, morphine has been shown to suppress the primary but not the secondary humoral immune response to cholera toxin in mice (19). These results are relevant in light of efforts to stimulate protective mucosal immune responses in AIDS vaccine recipients, a fraction of whom are heroin abusers.

The production of opioid peptides by leukocytes during inflammation (20, 21) and the existence of opioid receptors on cells of the immune system seem to have a physiological consequence relative to antinociception (22, 23). The production of endogenous opioids may also contribute to autoimmune phenomena which may develop as a part of the inflammatory response (24, 25). However, there does appear to be a mechanism in place to rapidly remove locally produced opioid peptides via aminopeptidases and ectopeptidases (26-28) which would considerably reduce the potential for pathological manifestations associated with the local production.

A considerable amount of evidence suggests that opioids administered *in vivo* modify the immune system indirectly. The HPA axis has been implicated in morphine-mediated suppression of primary humoral immune responses (29), Kupffer cell and splenic macrophage phagocytosis of sheep red blood cells (30), thymic hypoplasia (31), phorbol myristate acetate-stimulated increase in CD25⁺ expression on CD4⁺ T lymphocytes (32), and splenic natural killer (NK) activity (33). Morphine has also been shown to activate adrenergic processes in suppression of splenic NK activity (34, 35) and mitogen-induced splenocyte proliferation (36). Using opioid receptor agonists administered intraventricularly, the μ -selective ligand DAMGO but not the δ -selective agonist DPDPE or the κ -selective agonist U50,488 was found to suppress splenic NK activity through a naltrexone-sensitive pathway (37). Other recent studies

also suggest morphine-mediated effects on the immune system operate through central processes (38-40). However, there is an indication that the HPA axis is not necessarily involved in all opioid-mediated immunosuppressive effects since microinjection of opioid compounds into specific sites of the brain that result in a suppressive effect on immune function (e.g., lymphocyte proliferation or splenic NK activity) do not change the circulating levels of corticosterone (37, 38). The immunosuppressive effects of opioid agonists may be mediated through the central nervous system, however this does not preclude a role for direct *in vivo* effects of opioids on cells of the immune system. The overall effect of *in vivo* administration of opioids may be suppression due to central actions which override direct peripheral actions.

Collectively, these results provide convincing evidence for the local and systemic activity of opioids on immunocompetence and immune homeostasis. Definitive proof of the existence of opioid receptors on cells of the immune system has recently been provided through the cloning of these receptors. The fact that the receptors cloned from leukocytes are highly homologous with those identified in neural tissue substantiates the previous studies showing biochemical and pharmacological evidence for opioid receptors on cells of the immune system (1).

Molecular Characterization of Leukocyte Opioid Receptors

Each of the major opioid receptor classes have now been cloned from neuronal cells and fully sequenced. These proteins are 7 transmembrane receptors and share homology with the somatostatin receptor (41). Analysis of the δ -opioid (42, 43), κ -opioid (44-48) and μ -opioid (49, 50) sequences show a high degree of amino acid homology among the opioid receptors. Northern blot analysis has shown that the δ -opioid receptor mRNA varies in size between 1.4 and 9 Kb (42). In a similar fashion the κ -opioid receptor mRNA isolated from

brain tissue is quite large (5.2 - 6.0 Kb) (44, 48), as is the μ -opioid mRNA (>10 Kb) (51). The large size of these transcripts is due to a long 3' untranslated region in each case.

The genomic organization of the opioid receptors is now being clarified. Both the μ - and δ -opioid receptors appear to possess three exons with multiple transcription initiation sites identified (52, 53). The κ -opioid receptor was first reported to be composed of three exons (54), however, more recent reports have clearly established the existence of an additional exon 5' of the exon containing the translation start site (55-57).

In the course of the examination of cDNA clones while involved in the cloning of the μ -, κ -, and δ -opioid receptors, several investigators have cloned and sequenced an opioid-like receptor which fails to bind to the opioid agonists commonly employed to characterize the classical opioid receptors (58, 59). This receptor possesses homology with the other opioid receptors and was originally termed the "orphan" opioid receptor. More recently the natural ligand for this receptor was identified as a heptadecapeptide which resembles dynorphin A and has been termed nociceptin (60, 61). The genomic structure of the nociceptin receptor is organized into three exons, and the translated sequence is approximately the same size as the cloned opioid receptors (54).

Efforts have recently been made to examine the molecular structure of the opioid receptors expressed by cells of the immune system. The evidence reviewed above suggested that the opioid receptors expressed in these cells may possess unique features. A partial sequence for the δ -opioid receptor was recently obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) from monkey lymphocytes (62). Examination of the partial sequence of this receptor revealed essentially complete identity with the brain δ -opioid receptor. A partial sequence for the μ -opioid receptor has also been reported for rat peritoneal macrophages (63) and monkey lymphocytes (64). Here again, the sequence is essentially

identical to the brain μ -opioid receptor. And finally, a partial κ -opioid receptor sequence has been obtained from human and monkey lymphocytes (65). The results from each of these studies strongly support the presence of classical opioid receptors expressed by cells of the immune system.

A recent study (57) represents the first report of a complete opioid receptor expressed by cells of the immune system. These investigators employed the immature T cell lymphoma cell line R1.1 as a source of mRNA for RT-PCR analysis and identified the presence of the κ -opioid receptor sequence (Fig. 1). This cell line has been shown previously to possess typical κ -opioid receptors based on pharmacological analyses (66-68).

RT-PCR was performed with RNA from the R1.1 cell line using several oligonucleotide pairs permitting analysis of the full-length sequence, as well as the sequence of individual regions of the open reading frame. Analysis of overlapping RT-PCR fragments and the full-length product revealed a surprising degree of heterogeneity at the 5' end of the coding region. Nucleotide sequences of multiple clones of the PCR products from the R1.1 thymoma cells were determined, and assembly of the individual overlapping PCR products and the sequence of multiple full-length clones confirmed the heterogeneity in the region of the κ -opioid receptor near the start codon. Clones were observed with a sequence which is essentially identical (99.8% nucleotide homology; 100% amino acid homology) to that of the reported murine brain κ -opioid receptor (44). Clones were also identified with a 30-bp insertion 15 bp upstream of the initiation codon. This 30-bp insertion is present in the cDNA of the rat brain κ -opioid receptor (45).

The presence of the 30-bp insertion in the 5'-noncoding region were somewhat surprising given the initial reports of the genomic structure of the mouse μ -, κ -, and δ -opioid receptors (52-54). Within the coding regions, there are two introns in the δ and κ receptors

and three introns in the μ receptor. The 30-bp insertion suggested alternative intron-exon splice variation which could only occur if an additional exon 5' of the exon containing an upstream transcription initiation site is present in the genomic sequence. More recent reports have clearly identified the sequence of this fourth exon in the κ -opioid receptor (54).

These results suggest that multiple κ -opioid receptor mRNA species are present in the R1.1 cell line, and either the κ -receptor or the cell population may be unique in this regard. The impact of these inserted sequences in the 5'-noncoding region on expression of the κ -opioid receptor remains uncertain at this time. Additional analyses of other cell lines as well as primary cells to determine whether any differences are seen in the sequence of the opioid receptors of these cells may provide more information on the interaction seen between the brain and immune system.

Further analysis of the R1.1 cell line has resulted in the identification of an additional intron-exon splice variant (Belkowski, S., Alicea, C., Zhu, J., Liu-Chen, L.-Y., Eisenstein, T., Adler, M., and Rogers, T., *submitted for publication*). This splice variant fails to utilize any part of the second exon for the mature transcript. This alternate splice variant was detected in the R1.1 cell line as well as primary macrophages and brain tissue. The level of expression of this truncated transcript relative to the full length transcript appears to vary among tissues. The primary macrophages appear to express predominantly the truncated transcript, while the brain expresses primarily the full-length transcript. These splice variants may provide a source of protein heterogeneity and may provide the basis for some of the unusual binding properties by the immune cells.

The nociceptin (or orphan opioid) receptor has also been cloned by RT-PCR from mouse T cells (Fig. 2, 69). In comparison to previously characterized orphan opioid receptor cloned from rodent brain cDNA libraries (58), the lymphocyte orphan opioid receptor has 100

% homology. Interestingly, a splice variant of the 1.5 kb lymphocyte opioid receptor (missing 15 bases between 378-392) was also identified in the brain receptor. However, the splice variant occurs in 45-50% of the orphan opioid receptor species in lymphocytes whereas the variant occurs in only 25-30% of the receptor species in brain. This alternative splicing may have important implications in intracellular signalling cascades since the missing amino acids constituting that portion of the first intracellular loop of the receptor may constitute a portion of tyrosine antigen recognition activation motif (69). An additional splice variant in this receptor has been identified in rat brain (50). In this case the alteration would be expected to yield a change in the third extracellular loop. The impact of these changes in the receptor sequence on the binding or signaling properties remains to be determined.

To explore the potential biological significance of the lymphocyte orphan opioid receptor, antisense experiments were carried out measuring *in vitro* polyclonal antibody production, lymphocyte proliferation, the generation of cytotoxic T lymphocytes (CTLs) in one-way mixed lymphocyte cultures, and IL-6 production by activated macrophages. The results show selective effects on the immune parameters measured (Table 1). Specifically, orphan opioid receptor antisense oligonucleotides suppressed lipopolysaccharide (LPS)-induced lymphocyte proliferation and polyclonal IgM production (69, Table 1) but there was no observable effect on the generation of CTLs or LPS-elicited IL-6 production by macrophages (Table 1). Given the recent identification of an endogenous ligand for the orphan opioid receptor (60, 61) termed nociceptin (60) which elicits a state of hyperalgesia, future studies on the role of this receptor (which is upregulated following mitogen-induced activation of lymphocytes.[69]) in the immune system seems warranted.

The use of the recombinant opioid receptors expressed in transfected cell lines has permitted a more precise examination of the binding properties of the cloned receptor classes. In each case, however, the pattern of agonist and antagonist sensitivity is consistent with the

established pharmacological behavior of these receptors (41). The availability of these transfected cells should permit a more extensive analysis of the signal transduction mechanisms following receptor activation.

The relationship between opioids and infection

The concept that opioids have a significant biological role in modifying the immune system and thus, predisposing opioid users (e.g., heroin addicts) to infectious pathogens was not originally considered by physicians treating heroin addicts that presented with bacterial, protozoan, and viral infections in the early observations reported in the middle of this century (70-72). In fact, the increased incidence of infection was attributed to non-sterile needle use during the administration of the drug. Other studies noted that heroin addicts had immunologic abnormalities that may in part be due to the impurities in the acquired heroin or the result of the infection itself (73, 74). Currently, there is an active discussion as to the direct role of opioid exposure on acquiring infectious pathogens. Accordingly, this section reviews those studies that have used pharmacologically controlled conditions to evaluate opioid exposure and infection caused by microbial pathogens.

The immunosuppressive qualities of opioid alkaloids on phagocytosis by macrophages and neutrophils as well as antigen-specific antibody production by B lymphocytes (plasma cells) would logically lead one to predict that these drugs would have detrimental effects on the capacity of immune cells to clear bacterial and fungal infections. To this end, one study has found that resident peritoneal macrophages obtained from morphine pellet (75 mg)-treated mice have a significantly reduced phagocytic index capacity against *Candida albicans* as measured *in vitro* (4). These results support earlier work showing that *Candida albicans*-infected mice that were subsequently exposed to morphine (25 mg/kg, s.c.) 3, 24, and 48 hr post infection succumbed to the infection more rapidly and in greater numbers (75).

Interestingly, naltrexone pellet (30 mg) implantation in mice blocked the morphine-mediated suppression of the phagocytic index to *Candida albicans* (4) (suggesting the event was mediated through opioid receptors) whereas the administration of naloxone (1-4 mg/kg/day) had no effect on the mean survival time of morphine-treated mice infected with the pathogen (75). The discrepancy may be due to the amount of opioid antagonist employed, the form (pellet versus multiple injections) of morphine and opioid antagonist exposure, and the potency of the opioid antagonist. The immunosuppressive effect of morphine exposure *in vivo* on the phagocytosis and killing properties of macrophages and neutrophils measuring *C. albicans* may be time dependent since a recent report has shown an initial rise (20 min post morphine administration) and subsequent reduction (24 hr post morphine administration) in the killing capacity of peritoneal leukocytes on *C. albicans* organisms following an acute administration of morphine (20 mg/kg, s.c.) but not methadone (12.5 mg/kg, s.c.) administration (76). Transient changes in the killing capacity of polymorphonuclear cells measuring *C. albicans* blastospores have also been noted in morphine-treated (3-5 mg/kg, 3 times/day) rhesus monkeys (6).

Opioid influences on the incidence of viral infections in I.V. drug users has been well documented (77, 78). However, the polydrug use of the patient population as well as personal hygiene, adulterous drugs, and sharing of needles have all been considered in the explanation of the prevalence to viral infections in the I.V. drug use population (78). One of the earliest laboratory controlled studies showed that morphine pellet (50 mg) implantation of mice resulted in an increased incidence of encephalomyocarditis virus-induced death (79). The diminished resistance to the viral infection closely paralleled the suppression in viral-induced interferon production as well. Using Friend murine leukemia virus, another study showed that a single bolus of morphine (300 mg/kg, i.p.) given to the virally-infected mice substantially increased the mortality (100 %) whereas a chronic dosing regimen (10 - 100 mg/kg, i.p.) for 10 days (inducing a state of tolerance) prior to virus infection had no effect on the mortality incidence (80). In another study with this virus, it was found that the increased

incidence of mortality mediated by a single bolus of morphine (200 mg/kg, i.p.) was antagonized by the administration with naloxone (20 or 100 mg/kg, i.p.) at the time of or 5 min prior to morphine exposure (81). In this same study it was also noted that chronic administration of morphine (50 mg/kg/day over 9-21 days) had no effect on the mortality of mice infected with Friend virus even though there was a marked decline in spleen weight and a decrease in splenic viral titers. Although measurements on cell-mediated immune parameters (e.g., interferon production and NK and CTL levels) were not measured in this system, it would seem that these functions may not have been affected by the chronic administration of morphine. However, in another study assessing the effects of chronic morphine (50 mg/kg/day for 21 days, s.c.) administration on viral-induced death and modification of cell-mediated immunity, it was found that morphine exacerbated the encephalitic incidence of herpes simplex virus type 1 (HSV-1)-infected C3H/HeN mice (82). Likewise, chronic morphine exposure significantly suppressed the generation of CTLs in alloimmunized C3H/HeN mice. This effect was blocked by the μ -selective antagonist β -funaltrexamine (83) but not the δ -selective antagonist naltrindole (84) indicating the μ opioid receptor involvement in the suppression. Although there is a discrepancy as to the number of lymphocytes responding to alloantigen versus viral antigen, the effects of morphine on CTL generation may in part contribute to the deleterious effect of morphine on the immune response to HSV-1 infection.

In perhaps a more complete evaluation of immunocompetence under the duress of morphine tolerance, pigs immunized with bacterial and viral antigens were found to mount a humoral immune response to these antigens (85). However, the cell-mediated immune response as measured by delayed type hypersensitivity to 2,4 dinitrofluorbenzene was found to be diminished in the morphine-tolerant pigs. As a follow-up to this study, morphine tolerant pigs infected with swine herpes virus-1 and subsequently infected with *Pasteurella multocida* were found to display fewer neurologic signs and mortality associated with the encephalitis induced by the virus yet, exhibit significantly greater viral-induced and secondary bacterial

pneumonia (86). The protective effect against viral-induced encephalitis elicited by morphine is attributed to the anti-inflammatory action of the opioid on cell-mediated immunity which would explain the reduction in neuropathology typically associated with encephalitis (86).

The relationship between opioid use and viral infection/pathogenesis is portrayed most effectively with the primary causative agent of acquired immune deficiency syndrome (AIDS), human immunodeficiency virus type 1 (HIV-1). Given the high prevalence of HIV-1 infection among I.V. drug users and the ability of morphine to alter T lymphocyte and monocyte function, Peterson and colleagues investigated the *in vitro* effects of morphine on HIV-1 replication. Morphine was found to increase HIV-1 replication in human peripheral blood mononuclear cell (PBMC) HIV-1_{AT} PBMC cocultures as measured by p24 supernatant levels in a dose-dependent and β -funaltrexamine (μ -opioid selective antagonist)-sensitive fashion (89). Other investigators have made similar observations using heroin and measuring HIV-1 replication via syncytia formation in phytohemagglutinin-stimulated human PBMCs (90) and the monkey equivalent simian immunodeficiency virus (SIV) and measuring morphine modification of the cytopathic effects and RT activity in SIV-infected CEMx174 cells (89).

Thirty to fifty percent of the HIV-1-infected patients will develop neurological impairments including multinucleated giant cell encephalitis and vacuolar myelopathy. Within the central nervous system, HIV-1 appears localized within the macrophages and microglia (for review, 90). The entrance of HIV-1-infected monocytes into the brain has been predicted to be facilitated by secondary infections (e.g., bacterial or viral) that activate these monocytes into over expressing cytokines (TNF- α and IL-1 β) and thus, allowing the cells to marginate and subsequently diapedis through the endothelial lining into the brain parenchyma and perivascular regions of the brain (90). Similar to PMBCs, morphine has been found to amplify HIV-1 replication in chronically-infected promonocytes (U1) cocultured with human fetal brain cells (91). The mechanism of enhancement may involve TNF- α since other studies by this

group have shown that TNF- α production by microglia can enhance HIV-1 expression in the promonocytic clone U1 (92) and morphine can potentiate the production of TNF- α by LPS-stimulated microglial cells in a dose-dependent and naloxone/ β -funaltrexamine sensitive fashion (93). These results are consistent with earlier reports showing that TNF- α induces the expression of HIV-1 in chronically infected T cell clones (94) and membrane bound TNF- α stimulates HIV-1 gene expression (95). The observations showing the promotion/reactivation of HIV-1 replication by morphine calls into question the potential role of the endogenous opioids including dynorphin, [met]-enkephalin, and endorphins. Recently, β -endorphin has been shown to augment the replication and enhance the transactivation of the promoter of a neurotropic strain of HIV-1 (HIV_{JR-FL}) in fetal perivascular microglia through a naloxone-sensitive manner (96). Since the pro-opiomelanocortin derived peptide is typically secreted by the corticotrophs of the anterior pituitary following stress-induced corticotropin releasing hormone secretion of the hypothalamus, these results have important implications into the health and stress-related management of HIV-1-infected patients. Moreover, the reports of endorphin production by macrophages (97) might also play a crucial role in the neuropathogenesis of HIV-1 infection in the brain.

Although the relationship between opioids and infectious pathogens is most clearly demonstrated using viruses, morphine have been shown to modify the host response to parasitic infections including *Toxoplasma gondii* (98) and *Plasmodium berghei* (99). Moreover, a recent communication suggested that morphine pellet implantation facilitated the dissemination of indigenous microbial flora (specifically *Proteus mirabilis*) into the peritoneal fluid, spleen, and liver 24-48 hr post implantation in a naltrexone reversible manner (100). In addition, morphine was also found to sensitize mice to LPS-induced endotoxic shock suggesting that morphine may act as a co-factor in gram negative sepsis (100).

Summary

Our understanding of the impact of opioid compounds on the function of the immune system has expanded greatly over the past five years. It is now clear that several cell populations serve as targets for the effects of the opioids, and this includes T cells, macrophages, and NK cells. The mechanism(s) of immunomodulation are now being described in greater detail on both a cellular and biochemical level. Indeed, the finding that the production of lymphokines and cytokines may be altered following opioid treatment may be particularly important since all immune responses are dependent to some degree on the synthesis of these protein mediators.

The opioid receptors have now been successfully cloned from cells of the immune system. There is no longer serious doubt about the presence of opioid receptors expressed by these cell populations. Extremely valuable information regarding the role of the opioid receptors in the function of the cells of the immune system should be obtained using molecular methods. Clearly, the molecular basis for the effect of the opioid compounds on the immune response represents a critical area of research in the immediate years ahead.

It is not surprising that opioid compounds have been found to alter resistance to infectious agents since a great deal of evidence shows that these compounds modulate the immune response. The significance of the drugs of abuse in the host-parasite interaction for a number of microorganisms, including HIV, remains a critical area for additional research. In addition, because of the importance of opportunistic infections in the AIDS patient, the impact of opioids on the resistance to these infectious agents is also a matter of great concern. It is possible that combinations of certain drugs of abuse may serve to alter resistance to some, but not all, of these infectious diseases. In any case, answers to these questions will most

certainly come only once a greater understanding of the basic mechanisms of immunomodulation is achieved.

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Figure 1. Comparison of κ -opioid receptor sequences of R1.1 cDNA with the cDNA of brain cells: Nucleic acids which are identical to brain κ -opioid receptor are indicated by asterisks. The sequence numbering of Yasuda et al. (44) is used for the brain cDNA sequence. The R1.1A sequence is 98% homologous to the brain sequence. The R1.1B sequence contains 30 bp in the region 5' of the ATG translational start codon. Reprinted with permission of the authors and the publisher (57)

Figure 2. Proposed secondary structure of the orphan opioid receptor. Tyr⁷¹ - Arg⁷⁵ are shown in a different font to indicate that they may be absent in some orphan opioid receptors due to alternative splicing of mRNA. Cys¹²⁰ - Cys¹⁹⁸ shown in boldface type, are thought to form a disulfide bridge by which the first and second extracellular loops of the orphan opioid receptor are covalently bound.

**TABLE I. Orphan Opioid Receptor Antisense Oligonucleotide
Effects on Selected Immune Parameters^a**

IMMUNE PARAMETER TESTED	CONCENTRATION RANGE OF ANTISENSE OLIGO	EFFECT
CTL generation ^b	0.5 - 5.0 μ g/ml	no effect
IL-6 production ^c	1.0 μ g/ml	no effect
Lymphocyte proliferation ^d	1.0 μ g/ml	50 % suppression
Polyclonal antibody production ^e	1.0 - 5.0 μ g/ml	50 - 70 % suppression

^a Antisense oligonucleotide was prepared against the 5' untranslated region of the lymphocyte orphan with the sequence 5'-AGCCACTCAGTACAGTTC-3'. As a control, a scrambled sequence of the orphan opioid receptor was generated as well (i.e., 5'-ATCCCTTAATCGCGCAAG-3'). The antisense and scrambled oligonucleotides were administered to cultures *in vitro* at the time of culture or up to 24-hr prior to the activation of cells.

^b A one-way mixed lymphocyte reaction was initiated using 6×10^6 C57BL/6 (H-2^b haplotype) splenic lymphocytes and 4×10^6 DBA/2 (H-2^d haplotype) irradiated (900 rads) splenic lymphocytes in 2 ml of media. The oligonucleotides were added at the initiation of culture. Following a 5 day incubation period, the cells were harvested and assayed for CTL activity using P815 (H-2^d haplotype) in a 4-hr ⁵¹Cr-release microcytotoxicity assay.

^c Peritoneal macrophages were cultured for 18-24 hr and 1.0 μ g/ml of oligonucleotide along with LPS was added. Following an 18-hr incubation period, supernates were collected and assayed for IL-6 production by ELISA. In some experiments, the oligonucleotides were incubated with the cells for 24 hr prior to the addition of the stimulus (LPS).

^d and ^e See reference 69 for details.

brain	TGGAAAGCTGACGGTGACTTGGGAAGGGAGGTCGCCAATCAGCGATCTGGA-----	170
R1.1A	*****	170
R1.1B	*****GCCCCGCAAGTGCC	184
brain	-----GCTGCAGCGCTCACCATGGAGTCCCCATTTCAGATCTTCCGAGGAGATC	219
R1.1A	*****	219
R1.1B	ACCTTCTCGCTTTCCA*****	249
brain	CAGGCCCTACCTGCTCTCCAGTGCTTGCCCTTCTCCCAACAGCAGCTCTGGTTCCCAACTGG	284
R1.1A	*****	284
R1.1B	*****	314
brain	GCAGAATCCGACAGTAATGGCAGTGTTGGGCTCAGAGGATCAGCAGCTGGAGTCCGCGCACATCTC	349
R1.1A	*****	349
R1.1B	*****	379
brain	TCCGGCCATCCCTGTTATCATCACCGCTGTCTACTCTGTGGTATTTGTGGTGGGCTTAGTGGGCA	414
R1.1A	*****	414
R1.1B	*****	444
brain	ATTCTCTGGTCATGTTTGTTCATCATCCGATACACGAAGATGAAGACCGCAACCAACATCTACATA	479
R1.1A	*****	479
R1.1B	*****	509
brain	TTTAACCTGGCTTTGGCAGATGCTTTGGTTACTACCACTATGCCCTTTCAGAGTGCTGTCTACTT	544
R1.1A	*****	544
R1.1B	*****	574
brain	GATGAATTCTTGGCCTTTTGGAGATGTGCTATGCAAGATTGTCATTTCCATTGACTACTACAACA	609
R1.1A	*****	609
R1.1B	*****	639
brain	TGTTTACAGCATATTCACCTTGACCAATGCAATGTCAGGCTACATGGTGTGTGCCACCTT	674
R1.1A	*****	674
R1.1B	*****	704
brain	GTGAAAGCTTTGGACTTCCGAACACCTTTGAAAGCAAAGATCATCAACATCTGCATTTGGCTCCT	739
R1.1A	*****	739
R1.1B	*****	769
brain	GGCATCATCTGTTGGTATATCAGCGATAGTCCCTGGAGGCACCAAGTCAGGGAAGATGTGGATG	804
R1.1A	A*****G*****	804
R1.1B	*****	834
brain	TCATGAATGCTCCTTGCACTTTCCTGATGATGAATATTCCTGGTGGGATCTCTTCATGAAGATC	869
R1.1A	*****	869
R1.1B	*****	899
brain	TGTGTCTTCGTCTTTGCCTTTGTGATCCAGTCTCATCATCATTGTCTGCTACACCTGATGAT	934
R1.1A	*****A*****	934
R1.1B	*****	964
brain	CCTGCGCCTGAAGAGTGTCGGGCTCCTGTCTGGCTCCCGAGAGAAGGACCGAAATCTCCGCCGCA	999
R1.1A	*****	999
R1.1B	*****	1029
brain	TCACCAAGCTGGTGCTGGTAGTAGTTGCAGTCTTCATCATCTGTTGGACCCCCATTTCATCTTT	1064
R1.1A	*****	1064
R1.1B	*****	1094
brain	ATCCTGGTGGAGGCTCTGGGAAGCACCTCCCAACAGCACAGCTGCCCTCTCCAGCTATTATTCTG	1139
R1.1A	*****	1139
R1.1B	*****	1169
brain	TATTGCCTTGGGTTATACCAACAGCAGCCTGAATCCTGTTCTCTATGCCTTTCTGGATGAAAAC	1194
R1.1A	*****	1194
R1.1B	*****	1224
brain	TCAAGCGGTGTTTATAGGACTTCTGCTTCCCTATTAAGATGCGAATGGAGCGCCAGAGCACCAAT	1259
R1.1A	*****	1259
R1.1B	*****	1289
brain	AGAGTTAGAAACACAGTTCAGGATCCTGCTTCCATGAGAGATGTGGGAGGGATGAATAAGCCAGT	1324
R1.1A	*****	1324
R1.1B	*****	1354
brain	ATGACTAGTCGTGAAATGTCTTCTTATTGTTCTCCAGGTAGAGAAGAGTTCA	1377
R1.1A	*****	1377
R1.1B	*****	1407

FIGURE 1

Mouse Orphan Opioid Receptor

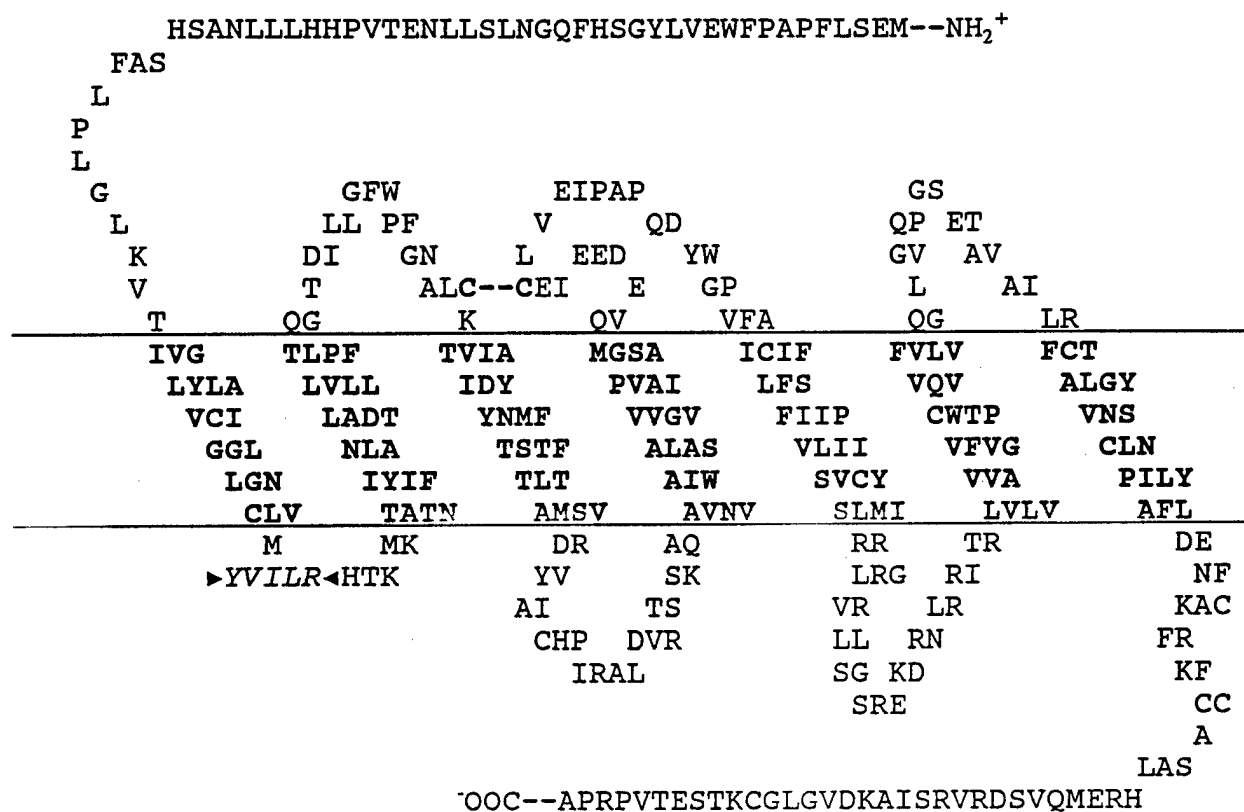


FIGURE 2

Persistent Cytokine Expression in Trigeminal Ganglion Latently Infected with Herpes Simplex Virus Type 1¹

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Following ocular infection, herpes simplex virus type 1 (HSV-1) establishes latency in trigeminal ganglion (TG) neurons. Using reverse transcription-PCR, cytokine gene expression was analyzed in the TGs of mice infected with HSV-1. IL-2, TNF- α , IFN- γ , IL-10, and RANTES mRNAs were readily detected in TGs taken from mice 7 days postinoculation (PI). Likewise, IL-2, IL-6, IL-10, and IFN- γ protein were detected by ELISA of TG homogenates. Between 5 and 45 days PI, IL-10, IFN- γ , TNF- α , and RANTES mRNAs were detected in nearly 100% of latently infected TGs (latent infection was confirmed by reverse transcription-PCR detection of HSV-1 latency-associated transcripts). T cell-associated cytokine and chemokine mRNAs (IL-2, IL-10, IFN- γ , and RANTES) were still detected in the majority of latently infected TG samples taken between 60 and 135 days PI. In contrast, these cytokine mRNA species were rarely detected in uninfected TGs. Measurement of serum Abs to HSV-1 at different times revealed that anti-HSV-1 Ab concentrations approached a plateau in mice by 30 days PI but remained at high levels 67 and 125 days PI. Although there was molecular evidence of an ongoing immune response to HSV-1 in latently infected TG, histologic analysis indicated that very few mononuclear cells remained in the ganglion 60 days PI. Collectively, the results suggest that residual lymphocytes encounter viral Ag during HSV-1 latency with sufficient frequency to remain activated. The paradox of a persistent immune response against a latent infection is discussed. *The Journal of Immunology*, 1996, 157: 0000–0000.

Following primary infection of the eye or oral mucosa, herpes simplex virus type 1 (HSV-1)³ is carried from the epidermis to the trigeminal ganglion (TG) by retrograde transport along sensory nerve fibers (1). Viral Ag expression peaks in the TG 3 days postinoculation (PI) and rapidly induces an inflammatory response and elevated cytokine expression in the TG (2). By 10 to 14 days PI, HSV-1 replication ceases as a host immune response is mounted, and latency is established. During latency, viral genomes are stably maintained in the neuronal nucleus as episomes that associate with cellular histones (3, 4). Viral transcription is generally thought to be limited to the expression of latency-associated transcripts (LATs) (5). Despite efforts to identify a functional open reading frame (6), a LAT protein has not been identified *in vivo*.

When viral replication spreads to the central nervous system before an effective immune response is mounted, the outcome of primary HSV-1 infection is fatal encephalitis. In susceptible strains

of mice, 5 to 10 days PI is the crucial period during which either the primary infection will be controlled or encephalitis will develop. Differences in the susceptibility of mouse strains (i.e., C57BL/6 and BALB/c) to herpetic encephalitis has been attributed to a general decrease in permissiveness of C57BL/6 mouse cells for HSV-1 replication (7). However, there are also reports that immune factors such as MHC I haplotype (8) and IFN production (9) are critical determinants in controlling acute HSV-1 infection. Regardless of the exact mechanism, it is important that an effective immune response is mounted before HSV-1 can spread unchecked into the central nervous system and cause encephalitis.

From 5 to 10% of TG neurons become latently infected with HSV-1 following ocular infection (10, 11). Despite the extent of neuronal infection, <2% of ganglionic neurons are destroyed during acute HSV-1 infection (12). Although there is some debate over the relative importance of CD4⁺ (13) and CD8⁺ (12) lymphocytes, T cells are generally considered to be essential for controlling HSV-1 replication and allowing the transition to latency (14–16). Because of the absence of extensive neuronal cell death, it appears that T lymphocytes shut down viral replication in the ganglion by noncytolytic mechanisms. Specifically, T cell-secreted cytokines are believed to contribute to the efficient establishment of latency by inducing a nonpermissive state in ganglion cells (12, 17). Cytokines, including IFN- α , IFN- γ , and TNF- α , have been implicated in limiting the pathogenesis of HSV following primary infection (18, 19).

If true latency of HSV-1 is established, cytokine expression in the TG should cease in the absence of viral Ag. However, several recent studies have found evidence of a lingering cell-mediated immune response in the TG of latently infected mice. Immunohistochemical studies have detected IFN- γ -positive cells 6 mo PI (20). Likewise, CD4⁺, CD8⁺, $\gamma\delta$ TCR⁺, and CD45RA⁺ lymphocytes are present in latently infected TG from 5 to 92 days PI, along with significant numbers of macrophages (F4/80⁺ cells) (2, 20, 21). In the present study, a cytokine response persisted in latently infected TG for up to 4 mo PI. Furthermore, serum anti-HSV-1 Ab concentrations in mice remained elevated 125 days PI.

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³ Abbreviations used in this paper: HSV-1, herpes simplex virus type 1; G3DPH, glyceraldehyde-3-phosphate dehydrogenase; ICP27, infected cell polypeptide 27; LAT, latency-associated transcript; TG, trigeminal ganglion; LAT⁺ TG, trigeminal ganglion samples with presence of latency-associated transcript confirmed by reverse transcription-PCR; LAT[−] TG, trigeminal ganglion samples with absence of latency-associated transcript confirmed by reverse transcription-PCR; pfu, plaque-forming unit; PI, postinoculation; RANTES, regulated upon activation, normal T cell expressed and secreted (mRNA); RT, reverse transcriptase.

Collectively, the data highlight the paradox of immune effector cells persisting at the site of a latent infection.

Materials and Methods

Virus and cells

The Vero and CV-1 African green monkey kidney cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 5% fetal bovine serum (Life Technologies, Gaithersburg, MD) and an antibiotic/antimycotic solution (Atlanta Biologics, Atlanta, GA). Cells were incubated at 37°C, 5% CO₂, and 95% humidity.

Mice were infected with HSV-1 (McKrae strain) that was grown as follows. Vero cells were infected with 0.1 to 0.5 plaque-forming unit (pfu) of HSV-1 per cell. After allowing 1 h at 37°C for viral adsorption, the medium was discarded, cell monolayers were rinsed with PBS, and culture medium was added. Infected cells were incubated at 34°C until the cytopathic effect was generalized (i.e., 48–72 h). Culture medium containing virus was centrifuged at 1000 × *g* for 10 min to remove cellular debris, and aliquots of the clarified supernatant were stored at –70°C.

Infection of mice

Female ICR mice (25–34 g; Harlan-Sprague-Dawley, Indianapolis, IN) were anesthetized by i.m. injection of ketamine (100 mg/kg) and i.p. injection of xylazine (10 mg/kg). Following corneal scarification with a 25-gauge needle, tear film was blotted from the eyes with tissue, and mice were inoculated with HSV-1 by placing 3 µl of a 10⁵-pfu/ml solution (i.e., 300 pfu) on each eye. Mice were given 0.1 ml of rabbit antiserum to HSV-1 i.p. at the time of infection to enhance survival (22). Infection was verified by swabbing the eyes 2 days PI, placing the swabs in CV-1 monolayer cultures, and observing the cultures for cytopathic effects. Animals were handled in accordance with National Institutes of Health Guidelines on the Care and Use of Laboratory Animals (23).

Reverse transcription-PCR analysis of TGs

To prevent contamination of TGs by cytokine-producing blood leukocytes, mice were killed by CO₂ asphyxiation and bled by cutting the superior vena cava before the skull was opened. Following dissection, TGs were rinsed in PBS and blotted on tissue paper to remove traces of blood. Left and right TGs were pooled and homogenized with a Pellet Pestle Motor (Kontes, Vineland, NJ) in Ultraspec RNA isolation reagent (Biotex, Inc., Houston, TX). RNA was extracted according to the manufacturer's instructions. First strand cDNA was synthesized from 4 µg of total TG RNA using an oligo-dT₁₅ primer and avian myeloblastosis virus reverse transcriptase (RT) (Promega Corp., Madison, WI) in a 40-µl reaction volume. Because the major species of LAT is not polyadenylated (24), 20 ng of a LAT-specific oligonucleotide (ICP0-3') was used as a primer in a separate RT reaction containing 500 ng of total TG RNA in a 10-µl reaction volume. Reverse transcription was performed according to the manufacturer's directions. TG cDNA (3.5 µl) was combined with 1× Taq buffer, 0.25 µM concentrations of each PCR primer, 100 µM concentrations of each deoxyribonucleoside triphosphate, and 2.5 U of Taq polymerase (Promega) in a 50-µl reaction volume and overlaid with mineral oil. Oligonucleotide primers used in this study were synthesized by the Louisiana State University Medical Center Core Laboratories (New Orleans, LA) and are listed in Table I. PCR was performed in an MJ Research thermal cycler (Watertown, MA) with 35 cycles of 94°C (1 min) → 57°C (1 min) → 72°C (30 min). PCR products were resolved in 2% agarose gels and were visualized by ethidium bromide staining in an EagleEye II still video system (Stratagene, La Jolla, CA). Densitometric analysis of gel images was performed using ImageQuant 3.3 software (Molecular Dynamics, Sunnyvale, CA).

ELISA of cytokine proteins in TGs

TGs from five mice were homogenized with a Pellet Pestle Motor (Kontes) in 1.0 ml of carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) containing 50 µg/ml bacitracin, 100 µg/ml aprotinin, 100 µg/ml pepstatin A, 100 µg/ml benzamidin, and 5 µg/ml PMSF. Following centrifugation (1 min at 14,000 × *g*), IL-2, IL-6, IL-10, IL-12, and IFN-γ concentrations in clarified homogenates were determined by ELISA (PharMingen, San Diego, CA). The protein concentration in clarified TG homogenates was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's directions.

Table I. Oligonucleotide primers

Primer	Product Size (Base Pairs)	Sequence
G3PDH-a	239	GAATCTACTGGCGTCTTCACC
G3PDH-b		GTCATGAGCCCTTCCACGATGC
ICP0-3'	–	TTCGACCAGGGGACCCTAGT
LAT-a	195	GACAGCAAAAATCCCCTGAG
LAT-b		ACGAGGAAACCAATAAGGG
ICP27-a	283	TTTCTCCAGTCTAGCTGAAGG
ICP27-b		TCAACTCGCAGACACGACTCG
IL-2-a	247	TCCACTTCAAGCTCTACAG
IL-2-b		GAGTCAAATCCGAACATGCC
IL-10-a	256	GGACAACATACTGCTAACCGAC
IL-10-b		AAAATCACTCTTCACTGCTCC
IFN-γ-a	237	AACGCTACACACTGCATCTTGG
IFN-γ-b		GACTCAAAGAGTCTGAGG
RANTES-a	271	GAAGATCTCTGCAGCTGCCCT
RANTES-b		GCTCATCTCCAAATAGTTGA
TNF-α-a	384	GCCTGTAGCCACGTCGTAG
TNF-α-b		TTGGGCAGATTGACCTCAGC

ELISA measurement of anti-HSV Ab titers

HSV-1 virion proteins were used as the coating Ag in ELISAs and were obtained as follows. Five 150-cm² flasks of CV-1 cells were infected with 5 pfu of HSV-1 per cell and incubated at 37°C for 24 h. HSV-1-infected cells were freeze-thawed three times in the culture medium. The cell lysate was clarified by centrifugation at 1,000 × *g* for 10 min. Virions were pelleted onto a 60% sucrose cushion by centrifugation (20,000 rpm, 4°C, 4 h) in a Beckman 28 SW.1 rotor (Beckman Instruments, ^{Palo Alto, CA} CA). Virions were collected from the culture medium/sucrose interface using a syringe. The virion-containing fraction was diluted 1:5 in PBS, and the virions were then further purified over a discontinuous sucrose gradient (i.e., the virion fraction was applied to a gradient containing layers of 15, 25, 35, and 60% sucrose that was then centrifuged at 4°C in a Beckman 28 SW.1 rotor for 4 h at 20,000 rpm). Purified virions were collected from the interface between the 35 and 60% sucrose layers using a syringe, and the virion-containing solution was dialyzed against PBS.

ELISA to determine anti-HSV Ab titers was performed as follows. EIA 96-well plates (Costar Corp., Cambridge, MA) were coated with 100 µl of HSV-1 virion proteins (diluted 1:50 in carbonate buffer) for 12 h at 4°C. Wells were blocked with 350 µl of 0.5% dry milk dissolved in PBS for 1 h at 37°C. After one rinsing with PBS, duplicate 100-µl samples of diluted mouse serum (1:50 dilution in PBS) were added to HSV-1 Ag-coated wells and incubated for 1 h at 37°C. After six rinsings with PBS (pH 7.4) containing 0.05% Tween-20 (polyoxyethylene-20-sorbitan monolaurate), 100 µl of affinity-purified goat anti-mouse IgG [H] and [L]-alkaline phosphatase-conjugated Ab (diluted 1:1500 in PBS; Bio-Rad Laboratories, Richmond, CA) was added to each well and incubated for 30 min at 37°C. After six rinsings with PBS/Tween-20, 200 µl of *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) was added to each well, and colorimetric development (OD₄₀₅) was measured in an ELISA plate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Statistics

Comparison of normally distributed data was performed by one-way ANOVA and Tukey's post hoc analysis. Comparison of nonparametric data was performed using Fisher's exact test. The correlation coefficient (of densitometric data) and its statistical significance were determined by regression analysis. In these statistical analyses, *p* < 0.05 was considered the minimum significant difference to reject *H*₀: *x*₁ = *x*₂.

Results

Cytokine expression in TG during acute HSV-1 infection

Six to nine days after infection with the neurovirulent HSV-1 McKrae strain, one-third to one-half of infected ICR mice succumb to fatal encephalitis. As viral replication spreads unchecked into critical areas of the brainstem and cerebellum (typically 24–36 h before death), mice begin to display visible symptoms of encephalitis, such as ataxia, hunched posture, and hypoexcitability.

Table II. Cytokine concentrations in TG homogenates^a

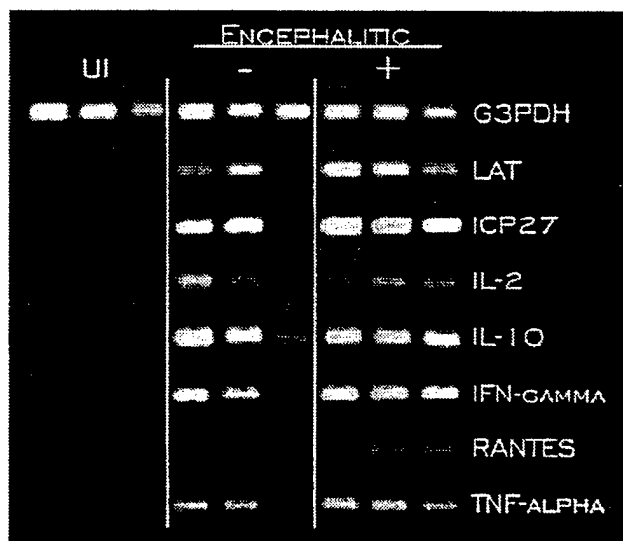
Cytokine Concentration	Nonencephalitic	Encephalitic
IL-2 (pg/ml)	331 ± 72	466 ± 59
IL-6 (pg/ml)	1021 ± 281	1201 ± 258
IL-10 (pg/ml)	126 ± 8	213 ± 21**
IFN-γ (U/ml)	ND ^b	17.5 ± 3.9*

^a Results represent mean ± SEM cytokine concentration (based on three to five independent experiments) as determined by ELISA of supernatants from the pooled homogenates of five pairs of TG.

^b ND = not detectable (i.e., <10 U/ml).

* Signifies $p < 0.05$. ** Signifies $p < 0.01$.

A



B

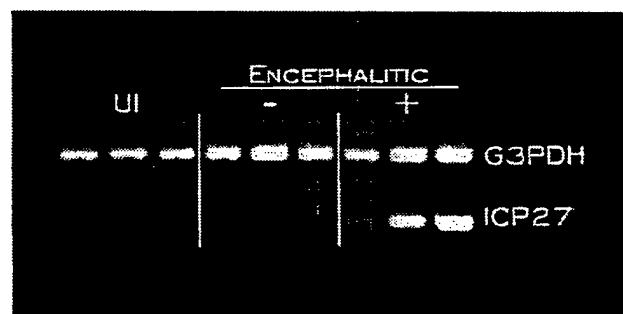


FIGURE 1. Cytokine transcription in acutely infected TG. **A**, Results of RT-PCR analysis of triplicate samples of TG from uninfected mice (UI) and either nonencephalitic (–) or encephalitic (+) mice 7 days PI. Top to bottom, RT-PCR was used to detect the following mRNAs in TG samples: the housekeeping gene *G3PDH*, HSV-1 *LAT*, HSV-1 *ICP27*, *IL-2*, *IL-10*, *IFN-γ*, *RANTES*, and *TNF-α*. **B**, Results of RT-PCR analysis of cerebellum samples from the same mice as in **A** for the presence of *G3PDH* and HSV-1 *ICP27* mRNA.

To determine whether the presence or absence of certain cytokines correlated with encephalitis, cytokine expression in the TGs of mice that did and did not show physical signs of encephalitis 7 days PI was compared. ELISA of TG homogenates demonstrated that IL-2, IL-6, IL-10, and IFN-γ protein were present in acutely infected TGs (Table II). However, IL-12 protein was not detectable by ELISA of TG homogenates. IL-10 and IFN-γ concentra-

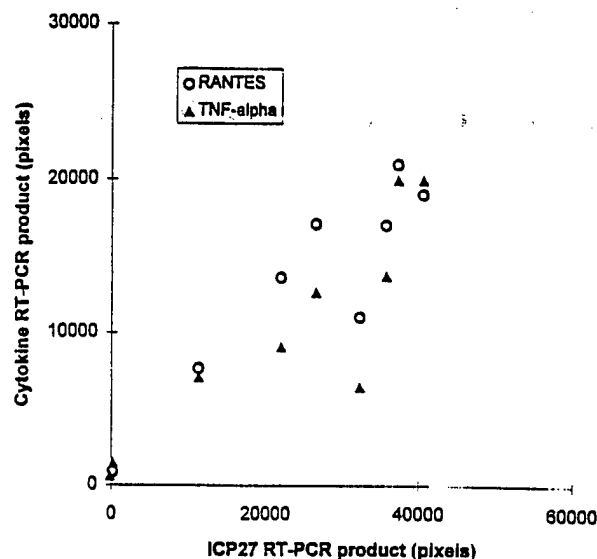


FIGURE 2. Correlation between cytokine and viral RT-PCR product yield amplified from HSV-1-infected TG. Each point represents the cytokine RT-PCR product yield vs ICP27 RT-PCR product yield amplified from a TG during the acute infection. Each pixel in the gel image is assigned a value between 0 and 255. RT-PCR product yield is therefore expressed in terms of the integrated volume of pixels associated with each ethidium bromide-stained band on an agarose gel.

tions were significantly higher in TGs of encephalitic mice relative to nonencephalitic mice 7 days PI (Table II). However, these differences were not evident at the mRNA level by RT-PCR analysis (Fig. 1A). Although there was no consistent correlation between HSV-1 lytic phase replication in the TG and encephalitis (Fig. 1A), encephalitis correlated well with HSV-1 replication in the cerebellum. Infected cell polypeptide 27 (ICP27) transcripts were abundant in the cerebellum of encephalitic mice but were not detected in the cerebellum of nonencephalitic mice 7 days PI (Fig. 1B).

Densitometric analysis of ethidium bromide-stained RT-PCR products indicated that a strong correlation existed between the yield of ICP27 and cytokine (i.e., *RANTES* and *TNF-α*) RT-PCR products amplified from TG 5 to 7 days PI (Fig. 2). The parallel increase in ICP27 and cytokine RT-PCR product yields was not due to differences in the quality of TG cDNA samples, because glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) RT-PCR product yield from these samples was very similar (i.e., 20,300 ± 650 pixels, mean ± SEM). The correlation coefficient between RT-PCR product yields of ICP27 and *RANTES* was 0.94 ($p = 0.0002$), and that between ICP27 and *TNF-α* was 0.89 ($p = 0.001$). Presumably, increased amounts of the cytokine mRNAs reflect a greater inflammatory infiltrate in the TG in response to larger virus loads.

Cytokine gene expression in TG during HSV-1 latency

Using RT-PCR, cytokine transcription was studied in the TG during the transition from acute to latent HSV-1 infection. RT-PCR detection of *LAT* RNA was used to confirm that TGs were infected with HSV-1 (Fig. 3). Because ICP27 is essential for productive HSV-1 infection, the absence of ICP27 mRNA confirmed that TGs were latently infected 14 days PI (Fig. 3). Although cytokine mRNA was not found in uninfected TG, cytokine transcript expression (especially *IL-10* and *IFN-γ* mRNAs) was strongly up-regulated in acutely infected TG 5 days PI (Fig. 3). *IL-2*, *IL-10*, *IFN-γ*, *RANTES*, and *TNF-α* mRNAs persisted in TG well after

FIGURE 3. Cytokine transcription in TG following HSV-1 infection. RT-PCR analysis of duplicate samples of TG taken from uninfected (UI) mice and from mice sacrificed 5, 14, 24, 35, 60, and 125 days PI. This figure is a representative example of RT-PCR experiments summarized in Table III. *Top to bottom*, RT-PCR was used to detect the following mRNAs in TG samples: the housekeeping gene *G3PDH*, HSV-1 *LAT*, HSV-1 *ICP27*, *IL-2*, *IL-10*, *IFN- γ* , *RANTES*, and *TNF- α* .

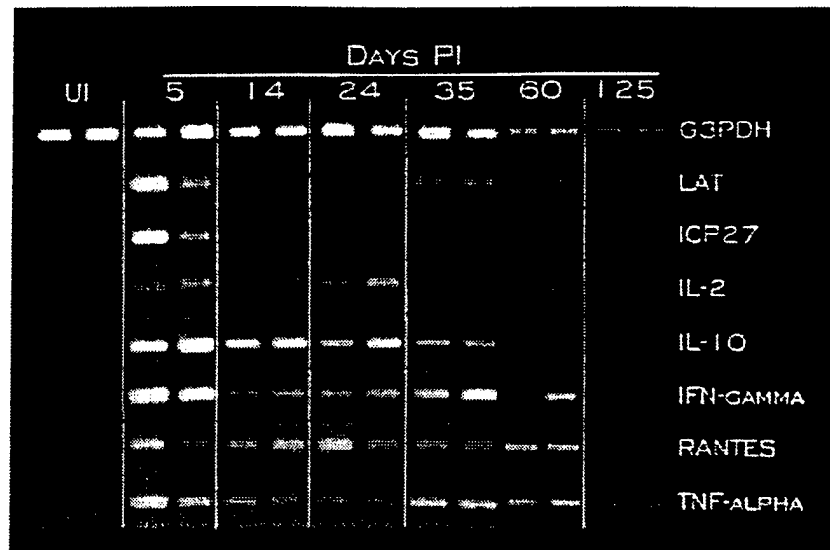


Table III. Cytokine transcription in LAT⁺ TG

Day PI	Cytokine-Positive TG (%) ^a				
	IL-2	IL-10	IFN- γ	RANTES	TNF- α
UI (<i>n</i> = 27) ^b	8 \pm 6	0 \pm 0	6 \pm 6	0 \pm 0	3 \pm 3
5-7 (<i>n</i> = 12) ^c	63 \pm 19	100 \pm 0	100 \pm 0	100 \pm 0	93 \pm 7
14-24 (<i>n</i> = 7) ^d	83 \pm 17	92 \pm 8	100 \pm 0	100 \pm 0	100 \pm 0
30-45 (<i>n</i> = 11-34) ^e	68 \pm 22	84 \pm 10	91 \pm 5	94 \pm 4	93 \pm 7
60-67 (<i>n</i> = 5-8) ^f	80 ^g	57 \pm 23*	80 \pm 10	70 \pm 30	80 \pm 10
110-135 (<i>n</i> = 10-15) ^h	38 \pm 5	63 \pm 3*	56 \pm 21*	52 \pm 21*	67 \pm 24

^a Results represent the mean \pm SEM percentage of cytokine transcripts in LAT⁺ TG as determined by RT-PCR.

^b Pooled results of nine experiments. UI = Uninfected.

^c Pooled results of three experiments.

^d Pooled results of three experiments.

^e Pooled results of seven experiments.

^f Pooled results of two experiments.

^g Result of a single experiment.

^h Pooled results of four experiments.

* *p* < 0.05, determined by ANOVA and Tukey's post hoc analysis comparing latent to acute TG (5-7 days PI).

active HSV-1 replication had ceased; cytokine transcripts were detected in TG 14, 24, 35, 60, and 125 days PI (Fig. 3).

In total, cytokine expression was evaluated in 27 uninfected mice and 91 HSV-1-infected mice killed between 5 and 135 days PI (Table III). The presence of LATs was confirmed in $84 \pm 5\%$ of infected TG samples by RT-PCR (hereafter referred to as LAT⁺ TG). When appropriate measures were taken to prevent contamination of TG with blood during dissection (see *Materials and Methods*), cytokine mRNAs were rarely detected in TG from uninfected mice (Table III). In contrast, IL-10, IFN- γ , RANTES, and TNF- α mRNA were detected in nearly 100% of LAT⁺ TGs between 5 and 45 days PI (Table III). The frequency of detecting IL-10, IFN- γ , and RANTES mRNA in LAT⁺ TG decreased significantly by 110 to 135 days PI, but there was still a more than 50% correlation between latent HSV-1 infection and the presence of cytokine transcripts (Table III). Expression of IL-10, IFN- γ , RANTES, and TNF- α mRNA was highly correlated. Typically, either all four or none of the cytokine mRNAs were detected in LAT⁺ TG. The correlation between the presence of IL-2 mRNA and the other cytokine mRNAs was more variable.

Comparison of LAT⁺ and LAT⁻ TGs

Despite the fact that primary infection was verified in mice by ocular swabbing 2 days PI, LAT RNA was not detected in 16% of

TG samples from infected mice. There was a strong correlation between detection of LAT and the presence of cytokine mRNA in TG (Fig. 4). Because there was no difference in the amount of G3PDH PCR product amplified from LAT⁺ and LAT⁻ TG (as determined by densitometric analysis), the failure to detect LAT and cytokine mRNA in LAT⁻ TG was not simply a result of differences in the quality of cDNA samples.

IFN- γ , RANTES, and TNF- α mRNA were detected significantly less frequently in LAT⁻ TG 5 to 14 days PI relative to LAT⁺ TG taken 5 to 14 days PI (Table IV). Whereas IFN- γ , RANTES, and TNF- α mRNA were detected in 75 to 80% of LAT⁺ TG taken 30 to 135 days PI, IFN- γ and RANTES mRNA were detected in none of the LAT⁻ TG taken 30 to 135 days PI. Likewise, TNF- α mRNA was detected in only 25% of LAT⁻ TG (Table IV). In contrast to these other cytokines, IL-10 was detected in four of five LAT⁻ TGs sampled 7 to 14 days PI and in three of eight LAT⁻ TGs sampled 30 to 135 days PI.

Results of ELISA detection of anti-HSV-1 Abs in serum from mice correlated with results of RT-PCR detection of LAT in TG. Although anti-HSV-1 Abs were found in serum from mice with TGs that were LAT⁺ by RT-PCR, serum anti-HSV-1 Abs were not detected in mice with TGs that were LAT⁻ by RT-PCR (Table V). Given this evidence that RT-PCR detection of LAT in TG was an

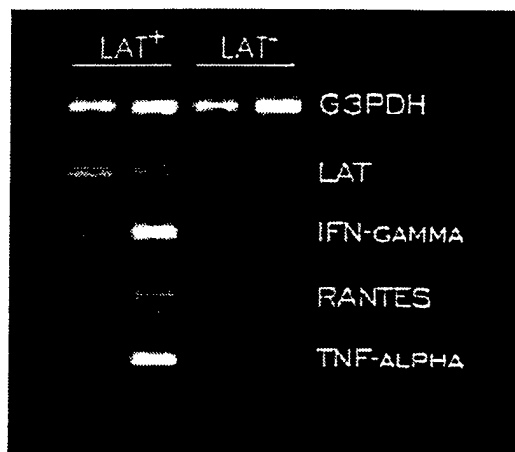


FIGURE 4. Comparison of cytokine mRNA profiles in LAT⁺ and LAT⁻ TG. RT-PCR analysis of representative TG samples taken from mice 37 to 42 days PI. Top to bottom, RT-PCR was used to detect the following mRNAs: the housekeeping gene *G3PDH*, HSV-1 LAT, IFN- γ , RANTES, and TNF- α .

Table IV. Cytokine mRNA detection in LAT⁺ and LAT⁻ TG

Days PI	LAT ^b	Fraction of Cytokine-Positive TG ^a				
		IL-2	IL-10	IFN- γ	RANTES	TNF- α
7-14	+	8/12	12/12	12/12	12/12	12/12
	-	1/5	4/5	2/5*	1/5**	1/5**
30-135	+	13/26	40/53	30/40	43/57	28/34
	-	1/4	3/8	0/8***	0/10***	2/8**

^a Results are expressed as concentration of cytokine⁺ TG per total number of TG.

^b Presence (+) or absence (-) of HSV-1 LATs as determined by RT-PCR.

* $p < 0.05$, determined by Fisher's exact test comparing LAT⁺ and LAT⁻ TG within either the 7 to 14 days PI group or the 30 to 135 days PI group. ** $p < 0.01$, determined as above. *** $p < 0.001$, determined as above.

accurate indicator of latent HSV-1 infection, the difference in cytokine mRNA profiles between LAT⁺ and LAT⁻ TGs was not unexpected.

Anti-HSV-1 Ab titers do not decrease during HSV-1 latency

After the acute infection, serum concentrations of anti-HSV-1 Abs increased steadily in mice from 7 to 30 days PI and reached a plateau by 50 days PI (Fig. 5). In the absence of viral Ag during latency, serum anti-HSV-1 Ab titers should peak after resolution of the acute infection and gradually decline. However, serum Abs against HSV-1 virion proteins were still present in high concentrations in mice 67 and 125 days PI (Fig. 5). The relative anti-HSV-1 Ab concentration in serum samples was derived by fitting sample OD values to a standard curve based on a twofold dilution series of high-titer anti-HSV-1 mouse serum ($r^2 = 0.995$). Based on the calculation, serum concentrations of anti-HSV-1 Abs were 1.5-, 3.1-, and 4.9-fold higher on average at 21, 50, and 125 days PI, respectively, relative to serum taken 14 days PI.

Histology of latently infected TG

The histology of TG sections from mice killed 14, 30, 62, 108, 121, and 135 days PI was compared with that of uninfected TG. Increased numbers of IFN- γ ⁺, IL-10⁺, and TNF- α ⁺ cells were evident by immunohistochemical staining of HSV-1-infected TG samples taken between 14 and 30 days PI, relative to uninfected control sections (data not shown). However, few differences were

Table V. ELISA of serum anti-HSV-1 Abs

Days PI	Anti-HSV-1 Ab ^a	
	LAT ⁻	LAT ⁺
0 ^b	0.031 \pm 0.009	—
35 ^c	0.022 \pm 0.013	0.277 \pm 0.068*
125 ^d	0.012 \pm 0.013	0.614 \pm 0.022**

^a Results represent mean \pm SEM OD₄₀₅ of anti-HSV-1 Ab ELISA on mouse serum. LAT⁺ = LAT RNA was detected in mouse TG by RT-PCR; LAT⁻ = LAT RNA was not detected in mouse TG by RT-PCR.

^b $n = 3$; 0 days PI indicates uninfected mice.

^c $n = 6$ per group.

^d $n = 3$ per group.

* $p < 0.01$, determined by ANOVA and Tukey's post hoc analysis comparing sample groups to OD₄₀₅ of serum from uninfected mice. ** $p < 0.0001$, determined as above.

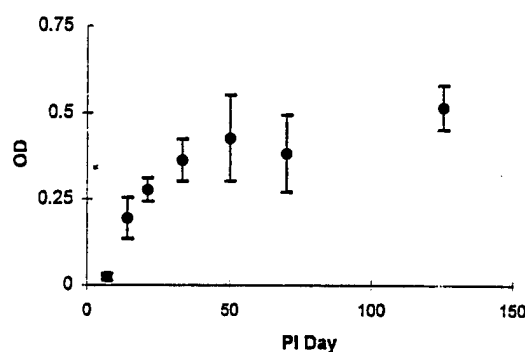


FIGURE 5. Anti-HSV-1 Ab concentrations at different times after infection. ELISA measurement of anti-HSV-1 Abs (OD₄₀₅) in mouse serum is shown vs time after inoculation (days PI). Results represent mean \pm SEM OD₄₀₅ and are based on serum obtained from mice 7 ($n = 4$), 14 ($n = 4$), 21 ($n = 20$), 33 ($n = 10$), 50 ($n = 5$), 70 ($n = 6$), and 125 ($n = 9$) days PI.

noted between uninfected TG and HSV-1-infected TG taken 62, 108, 121, and 135 days PI.

Relative to uninfected TG (Fig. 6A), an intense monocyte infiltrate was evident in TG 14 days PI during the resolution of acute HSV-1 infection (Fig. 6B). Likewise, areas of mononuclear cell infiltration were apparent among neuronal cell bodies in TG 30 days PI (data not shown). However, by 62 days PI histologic evidence of an ongoing immune response was difficult to find. Of 16 TG samples examined between 62 and 135 days PI (i.e., four TG per time point), only one neuron in a TG sample taken 108 days PI appeared to be the target of a mononuclear cell infiltrate (Fig. 6C). The focal nature of the infiltrate suggests an immune response to a neuron producing viral Ags. Although this point is speculative, such an infrequent, localized event may account for the persistence of cytokine-expressing cells in latently infected TG (4 mo PI), suggesting that a small population of lymphocytes is stimulated by viral Ag during latency.

Discussion

Cytokine expression during acute HSV-1 infection

The correlation between the presence of HSV-1 transcripts (i.e., ICP27 or LAT) and cytokine transcripts (i.e., IL-10, IFN- γ , RANTES, and TNF- α) in the TG was nearly 100% during resolution of the acute infection. The observation that T cell-depleted mice do not survive primary HSV-1 infection (25) demonstrates the importance of these effector cells and their secreted cytokines in controlling the primary infection. Cytokines, including IFN- α ,

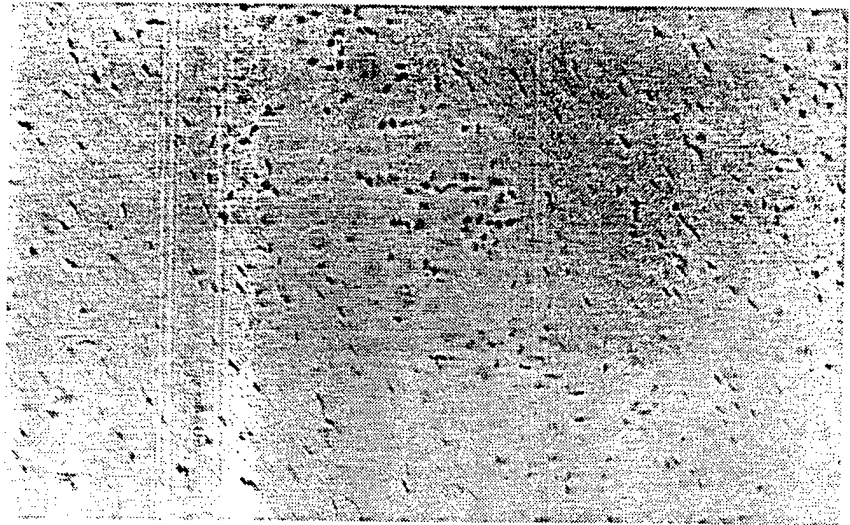
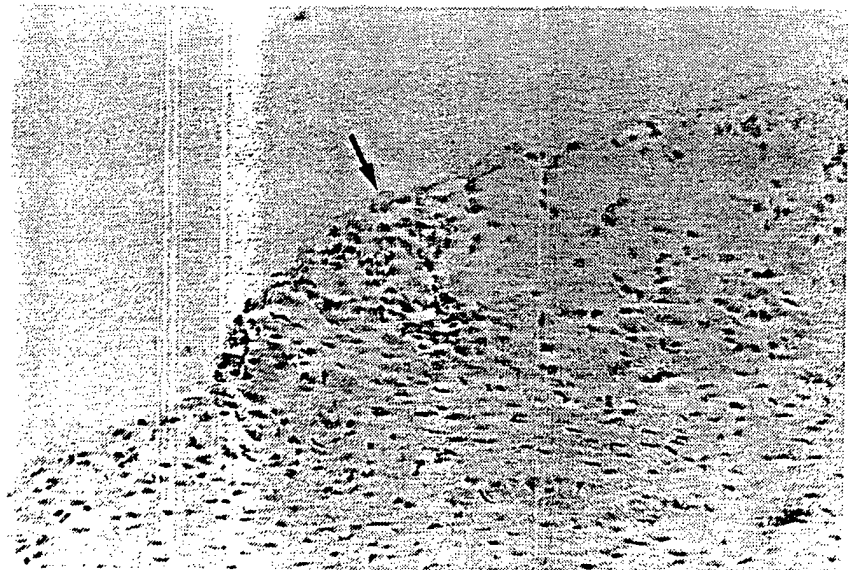
**A**

FIGURE 6. Mononuclear cell infiltration in TG. Hematoxylin-eosin-stained 8- μ m sections (20 \times magnification) of (A) an uninfected TG, (B) HSV-1-infected TG (14 days PI), and (C) TG latently infected with HSV-1 (108 days PI). Arrows point to areas of mononuclear cell infiltration. Histologic analysis was performed on four TG at each time point (14, 30, 62, 108, 121, and 135 days PI). To obtain a representative view of the TG, at least 12 sections (four adjacent sections from three different planes within the TG) were analyzed.

**B****C**

IFN- γ , and TNF- α , may also act directly on ganglion cells to block HSV-1 replication by inducing a nonpermissive state (26–28). This type of cytokine-mediated control of viral replication has been demonstrated in hepatitis B virus transgenic mice. IFN- γ and TNF- α produced by hepatitis B virus-specific CTLs block viral gene expression by inducing the selective degradation of hepatitis B virus mRNA in hepatocytes (29–31).

Cytokine expression in latently infected TG

The present study demonstrates that TNF- α and T cell-associated cytokine transcripts (IL-10, IFN- γ , and RANTES) are consistently present in latently infected TG 1 to 2 mo PI. Cantin et al. (20) observed large numbers of IFN- γ ⁺ cells 180 days PI in latently infected TG by immunohistochemical staining. In contrast, Liu et al. (2) observed only a few IFN- γ ⁺, IL-4⁺, and IL-10⁺ cells in latently infected TG taken 30 and 92 days PI but found many TNF- α ⁺ cells at these later time points. Our results are more consistent with the latter study, indicating that T cell cytokine expression persists well into latency but occurs only at low levels after 30 to 45 days.

The observation that T lymphocytes persist in latently infected TG has now been reported in A/J, BALB/c, and National Institutes of Health/OLA mice infected with HSV-1 strains RE, F, and McKrae, respectively (2, 20, 21). In the present study, outbred ICR mice were infected with HSV-1 (McKrae). Therefore, the persistence of T cells and cytokine expression in HSV-1 latently infected mouse ganglia is not a strain-specific phenomenon. However, this event may be unique to mice because T cells are not detected in rabbit TG 30 days PI by immunohistochemical staining (32).

Based on the use of immunosuppressive treatments (i.e., x-ray irradiation, cyclophosphamide) to induce reactivation from latently infected mice (33), one could argue that cytokine expression is necessary for the maintenance of HSV-1 latency in the TG. However, because of the systemic stress induced by these treatments, one cannot be sure that reactivation strictly follows from the immunosuppression. Furthermore, in an in vitro HSV-1 reactivation model, spontaneous reactivation does not occur from latently infected TG neurons (34) despite the absence of IFN- γ and little to no TNF- α in culture supernatants.⁴ Therefore, while cytokine expression appears to be important in controlling primary HSV-1 infection (20), the role of cytokines during latency remains unclear.

Implications regarding the nature of HSV-1 latency

The nature of HSV-1 latency has long been debated. It has been suggested that there may be a "trickle effect" such that low level HSV-1 replication occurs in latently infected TG. However, infectious virus is only infrequently recovered from tear film and TG of latently infected mice (35, 36). Therefore, HSV-1 is believed to establish a truly latent infection in mice in which spontaneous reactivation rarely occurs.

Low-level expression of HSV-1 proteins during latency could account for lymphocyte infiltration and T cell activation (i.e., cytokine expression) in latently infected TG. Immunoreactive ICP4 has been identified in latently infected rabbit TG (37), and, more recently, RT-PCR has been used to demonstrate low level ICP4 and HSV-1 thymidine kinase mRNA expression in latently infected mouse TGs (38). Likewise, LATs may encode an as-yet unidentified protein in vivo, such as the latency-associated Ag identified in vitro (39). However, latent HSV-1 has been detected

only in neurons (10, 40), a cell type that does not express MHC I (41) and is therefore incapable of presenting endogenous Ags to CD8⁺ T cells. Therefore, to accept the hypothesis that persistent cytokine expression in TG results from low-level viral Ag expression in latently infected neurons, one has to believe either that low-level MHC I expression occurs in neurons or that other Ag presentation pathways exist.

Other possible sources of Ag that could account for the persistence of T cells in latently infected TG are 1) self-antigens exposed during HSV-1 infection (42), 2) long-lived viral Ags that persist after the acute infection, 3) low level production of secreted viral Ags that are processed and presented by APCs (e.g., macrophages) to T lymphocytes, and 4) reactivation of infectious virus from latently infected neurons. Although HSV-1 reactivation seems a plausible source of Ag to account for T cell persistence in latently infected ganglia, infectious virus is only rarely detected in mouse TG after the acute infection (34, 35). Perhaps the explanation for this discrepancy is that periodic sampling for infectious virus is less sensitive than the host immune cells, which are continuously present. In the natural host (i.e., humans), HSV-1 efficiently reactivates in the face of humoral and cell-mediated immunity (43). Perhaps HSV-1 is unable to evade immune recognition during reactivation in mice, and so only small quantities of infectious virus are produced.

Regardless of the specific mechanisms, the data presented herein suggest that viral Ag is present in sufficient quantities during latency to stimulate an ongoing immune response. Further investigation is necessary to establish the underlying cause of this phenomenon.

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Cytokine Expression

in HSV-1 Latently Infected Trigeminal Ganglion Cell Cultures

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Abstract

Cytokine production was measured in primary cultures of trigeminal ganglion cells from both uninfected and herpes simplex virus type 1 (HSV-1) latently infected mice. Supernates from TG cell cultures contained detectable interleukin (IL)-6 but not IL-1 β , IL-2, IL-10, interferon (IFN)- γ , or tumor necrosis factor (TNF)- α . The basal level of IL-6 in uninfected TG cell cultures was 20.5 ± 2.3 ng/ml, whereas latently infected TG cells produced significantly less IL-6 (12.1 ± 1.9 ng/ml). Hyperthermic stress (43° C, 180 minutes), which induces reactivation of latent HSV-1 from TG cell cultures, significantly reduced IL-6 production from both uninfected and latently infected TG cell cultures. However, there was no correlation between the level of IL-6 production and HSV-1 reactivation in TG cell cultures. Dexamethasone, a glucocorticoid hormone analog, has been shown previously to induce HSV-1 reactivation in TG cell cultures. Dexamethasone (10^{-7} - 10^{-11} M) strongly inhibited IL-6 production in TG cells in a dose- and time-dependent manner. This inhibition occurred at the post-transcriptional level as determined by competitive RT-PCR. Likewise, at the highest dose tested, 10^{-6} M epinephrine suppressed IL-6 production 48-72 hr post treatment. Immunofluorescent studies indicated that cultured cells obtained from TGs contained 5-8% CD11b $^{+}$ cells, $0.9 \pm 0.4\%$ CD8 $^{+}$ cells, but contained no CD4 $^{+}$ cells. Collectively, the data suggest that IL-6 production in TG cell cultures does not play a role in hyperthermia-induced HSV-1 reactivation.

Introduction

Cytokine action and synthesis within the nervous system has been well documented (Fabris et al., 1994). IL-1 produced by microglial cells (Giulian and Lachman, 1986) has been shown to stimulate astroglial proliferation (Giulian et al., 1985). TNF- α synthesized by astrocytes (Liebermann et al., 1986; Chung and Benveniste, 1990) has been implicated as an important mediator in multiple sclerosis (Hofman et al., 1989). Likewise, IL-6 produced by microglial cells and astrocytes (Frei et al., 1989; Benveniste et al., 1990) has been associated with a number of disease processes related to infection (Frei et al., 1988; Frei et al., 1989) and autoimmunity (Gijbels et al., 1990). Consequently, the generation of cytokines by cells of the nervous system as well as the capability to respond to cytokines may have a significant impact on neural transmission (Besedovsky et al., 1983), the regulation of the hypothalamic pituitary adrenal (HPA) axis (Blalock, 1992), and the outcome of injury (Woodroffe et al., 1991) or infection (Nottet and Gendelman, 1995) in the brain or surrounding neuronal tissue.

HSV-1, a neurotropic virus that resides in the trigeminal ganglion following corneal infection elicits a vigorous immune response (Rinaldo and Torpey, 1993). Local control of the acute infection includes the presence of CD4⁺ and CD8⁺ lymphocytes (Nash et al., 1987; Bonneau and Jennings, 1989; Igietsme et al., 1991), neutrophils (Tumpey et al., 1996), IFN- α (Hendricks et al., 1991), and potentially IFN- γ and TNF- α (Cantin et al., 1995). Cytokine profiles following acute infection suggest that the cellular immune response is primarily of the TH1 type (Staats and Lausch, 1993; Bouley et al., 1995; Kanangat et al., 1996). However, the role of TH2 cells is more controversial. Specifically, the adoptive transfer of HSV-1 specific TH2 clones was shown to increase the onset and severity of stromal keratitis (Jayaraman et al., 1993) whereas the inoculation of the cornea with IL-10 (a TH2 cytokine) was found to reduce ocular inflammation as measured by cellular infiltration and IL-2 and IL-6 levels (Tumpey et al., 1994). The presence of these cytokines produced by the leukocytes infiltrating the TG and their

anti-viral, proinflammatory action suggest that cytokines are central to the control during the acute infection. Consistent with this hypothesis, disseminated neonatal HSV infection has a 50% mortality rate despite the presence of maternal anti-HSV antibodies in newborns (Whitley, 1993). However, the role of these cytokines in latent HSV-1 infection or in the reactivation of the virus from latency has not been addressed. It has been proposed that TNF- α (Liu et al., 1996) and IFN- γ (Cantin et al., 1995) may control reactivated HSV-1. The synergistic action of these cytokines in blocking HSV-1 replication supports this hypothesis (Feduchi et al., 1989; Chen et al., 1993). It is also thought that humoral immunity is involved in the maintenance of HSV-1 latency (Stevens and Cook, 1974).

Recently, an *in vitro* culture system has been developed (Moriya et al., 1994) and characterized (Halford et al., 1996a) to study HSV-1 reactivation from TG cells following hyperthermic stress. Primary cell cultures were established from dissociated TG of HSV-1 latently infected mice in the presence of an antiviral drug to block reactivation. Following removal of the antiviral compound 5 days after culture establishment, spontaneous reactivation did not occur in any cultures (n=52) monitored over a 20 day period. However, reactivation could be induced by hyperthermic stress or dexamethasone treatment. The present study was undertaken to identify cytokines produced locally that might be involved in HSV-1 latency and reactivation using this *in vitro* model.

Materials and Methods

Infection of mice. Female ICR mice (25-34 gm, Harlan-Sprague Dawley, Indianapolis, IN) were anaesthetized by intramuscular injection of ketamine (50 mg/kg) and intraperitoneal (i.p.) injection of xylazine (10 mg/kg). Following corneal scarification with a 25 g needle, tear film was blotted from the eyes with tissue, and mice were inoculated with HSV-1 (McKrae strain) by placing 3 μ l of a 10^5 pfu/ml solution (i.e. 300 pfu) on each eye. Mice were i.p. administered rabbit antiserum to HSV-1 at the time of infection to enhance survival [34]. Verification of primary infection was carried out by swabbing the eyes 2 days post-inoculation (PI), placing the swabs in CV-1 monolayer cultures, and observing these cultures for HSV-1 induced cytopathic effects.

Establishment of TG cell cultures.

TG cell cultures were prepared as previously described (Halford et al., 1996a). Briefly, TG were aseptically removed from latent HSV-1-infected mice (greater than 28 days PI). Single cell suspensions were obtained by incubating TG for 90 min at 37° C in calcium-, magnesium-free Hank's balanced salt solution containing 1 mg/ml collagenase type IV (Sigma Chemical Co., St. Louis, MO) and 1 mg/ml collagenase type IX (Sigma Chemical Co.). The suspension was triturated every 25-30 minutes to facilitate dissociation of TG. Dissociated cells were then washed (i.e. resuspended in 10 ml medium, and centrifuged for 5 minutes at 200 x g) twice with TG medium which consisted of minimum essential medium containing 10% fetal bovine serum (Gibco, Gaithersburg, MD), antibiotic/antimycotic solution (Sigma Chemical Co.), and 10 ng/ml nerve growth factor 2.5 s (Collaborative Biomedical Products, Bedford, MA). The cells were resuspended in TG medium containing 5 μ g/ml of (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU, Sigma Chemical Co.), and distributed into 24-well culture plates (Becton Dickinson Labware, Lincoln Park, NJ) that had been thin-coated with rat-tail collagen type I and recombinant mouse laminin (Collaborative Biomedical Products). Finally, culture plates were centrifuged (200 x g, 3

min) to promote cell adherence. Seven days following culture establishment, BVDU was removed from TG cells by replacing the culture medium. TG cells were maintained by replacing culture medium every 7 days. Cultures were incubated for 14 days before TG cells were heat stressed and / or culture supernatants were collected for cytokine measurements.

Drug treatment and cytokine measurements.

Cytokine concentrations were determined in the supernatants of TG cell cultures which either were, or were not, heat stressed. Twenty-four hours prior to heat stress, culture medium was replaced and each well received 2 ml of fresh TG medium. TG cells were heat stressed by placing culture plates in a 43°C tissue culture incubator (5% CO₂, 95% humidity) for 3 hours. Samples of culture supernatant were collected 12 hours prior to heat stress (basal measurement), immediately before heat stress, and 24, 48, and 72 hours after heat stress. Cytokine concentrations were determined by ELISA for IL-1 β (Genzyme, Cambridge, MA; Biosource Int., Camarillo, CA), IL-2, IL-6, IL-10, IFN- γ (Pharmingen, San Diego, CA) and TNF- α (Genzyme and Pharmingen).

In drug treatment experiments, culture medium was removed 24 hours prior to heat stress and replaced with medium containing either dexamethasone, epinephrine, or no drugs. Heat stress and collection of supernatants from drug-treated cultures was performed as described above.

Monitoring TG cultures for HSV-1 reactivation.

Infectious HSV-1 was detected by transferring 100 μ l of medium from TG cell cultures to monolayer cultures of CV-1 cells in 96-well plates (Costar, Cambridge, MA) which were then monitored for the appearance of cytopathic effect. TG cell cultures were sampled for infectious virus at 4, 8, and 12 days after culture establishment, immediately prior to heat stress, and were then sampled every 24 hour for five days after heat stress. The CV-1 African green monkey kidney cell line was obtained from the American Type Culture Collection (Rockville, MD).

Reverse transcription-PCR.

Total RNA was extracted from TG cells using Ultraspec™ RNA isolation reagent (Biotecx Inc., Houston, TX) as suggested by the manufacturer. First strand cDNA synthesis was carried out on equivalent amounts of RNA from each sample using an oligo (dT)₁₅ primer and AMV reverse transcriptase (Promega Corp., Madison, WI). PCR was carried out on equivalent amounts of template in a reaction buffer that contained 1x Taq buffer, 0.25 μM each oligonucleotide primer, 100 μM each dNTP, and 2.5 U Taq polymerase (Promega Corp) using a Thermolyne thermal cycler (Dubuque, IA) with 35 cycles of 94° C (1'15") → 57° C (1'15") → 72° C (20"). Oligonucleotide primers were synthesized by LSU Medical Center Core Laboratories (LSUMC, New Orleans, LA), purified by silica gel column and verified by polyacrylamide gel electrophoresis. Sequences used for the primers are shown in Table 1. Densitometry of ethidium bromide-stained agarose gels was carried out with an Eagle Eye II digital image capture system (Stratagene, La Jolla, CA).

Competitive RT-PCR.

Competitive PCR was performed on 5 μl of cDNA template in a 50 μl reaction volume containing 0.25 μM each oligonucleotide primer as previously described (Garza et al., 1996). Briefly, competitive PCR samples were subjected to 30 cycles of 94° C (1') → 57° C (1' 15") → 72° C (20"). Copy equivalents were determined by PCR of limiting dilutions, the lowest dilution in which amplification occurs being 1 copy equivalent. A 5 μl aliquot of each sample of cDNA product from the RT reaction was mixed with 5 μl of a predetermined amount (100 copy equivalents) of competitive mimetic standard. A competition for PCR primers was established where the cDNA in the greatest amount was amplified to yield a product of greater quantity. Standards were run in parallel using a known amount of cDNA (1-400 copy equivalents) and mimetic standard (100 copy equivalents) to generate a standard curve and determine the copy equivalents of each unknown sample. PCR products were electrophoresed on 2% agarose

gels containing 0.5 μ g/ml ethidium bromide and amplification of each sample was quantitated by its fluorescent intensity using the Eagle Eye II digital image capture system and ImageQuant 3.3 densitometry software (Molecular Dynamics; Sunnyvale, CA).

Fluorescence activated cell sorter (FACS) analysis.

Cells from TG cultures were obtained via trituration of the monolayers using ice-cold phosphate buffered saline (PBS, pH 7.0) containing 0.5% bovine serum albumin (BSA, Sigma). Cells were washed in PBS by centrifugation (250 x g, 5 min), the supernate discarded, the cells briefly vortexed, and fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibody to CD4, CD8, or CD11b (Pharmingen) applied to the dispersed cells at a concentration recommended by the manufacturer. Cells were incubated 20-30 min on ice in the dark and subsequently washed with PBS-BSA by centrifugation (250 x g, 5 min). Cells were resuspended in PBS containing 1% paraformaldehyde and analyzed on a Coulter Elite FACS (Coulter, Hialeah, FL). Log forward scatter vs. log side scatter plot was assembled to gate viable cells for analysis in order to separate cells from cellular debris. Light scatter was collected at 488 nm and emitted light was passed through a long pass filter followed by a narrow band filter and analyzed at 525 nm (FITC) and 575 nm (PE). Five thousand gated events were collected and analyzed per sample. Isotypic controls (Pharmingen) were used to subtract non-specific labeling of cells.

Statistics.

One-way analysis of variance (ANOVA) was used in conjunction with Tukey's post hoc t-test to determine significance ($p < .05$) between vehicle- and drug-treated groups using the GBSTAT program (Dynamic Microsystems Inc., Silver Springs, MD).

Results

Phenotype of TG cells in culture. Based on the number of cells expressing neuron-specific enolase, there are approximately 400-500 neurons per well in TG cell cultures (Halford et al., 1996a). However, the makeup of hematopoietic-derived cells in these cultures has not previously been characterized. TG of latently infected mice harbor infiltrating leukocytes including CD4⁺ and CD8⁺ lymphocytes as well as neutrophils (Cantin et al., 1995; Liu et al., 1996; Shimeld et al., 1995) and resident (microglia) and infiltrating macrophages (Shimeld et al., 1995). TG cell cultures were phenotypically characterized for the presence of CD4-, CD8-, and CD11b (Mac-1)-positive cells. Both latently infected and uninfected TG cell cultures possessed CD11b⁺ cells (Table 2). In addition, 4 of the 5 cell cultures derived from HSV-1 latently infected TG contained CD8⁺ cells (Table 2, Figure 1). However, there were no detectable CD4⁺ cells in either latently infected or uninfected TG cell cultures suggesting a minimal amount of T lymphocyte involvement in the TG cell cultures (Table 2).

Overview of cytokine expression in TG cell cultures. To identify cytokines produced by HSV-1 latently infected TG cells, culture supernates were assayed by ELISA for IL-1 β , IL-2, IL-6, IL-10, TNF- α , and IFN- γ . Collection of culture supernatants began 14 days after culture establishment. Samples were taken just prior to heat stress (i.e. 0 hour), and 24, 48, and 72 hours after heat stressing TG cells. Neither IL-1 β , IL-2, IL-10, nor IFN- γ were detected in any samples. However, IL-6 was consistently present in TG cell culture supernatants in high concentrations (10 - 300 ng / ml). A fraction of culture supernatants also contained low levels of TNF- α (100 -400 pg / ml). TNF- α was detected in 4 / 24 TG cell culture supernatants just prior to heat stress (i.e. 24 hours after replacing culture medium), and in 3/10 non-stressed controls at the same time point. At 24, 48, and 72 hours after heat stress, TNF- α was detected

in 0, 25, and 8% of heat-stressed TG cell cultures (n=24). In contrast, 0% of non-stressed controls (n=10) contained detectable amounts of TNF- α (less than 100 pg / ml) at these later time points.

TG cell cultures were assayed for HSV-1 reactivation following heat stress (i.e. the appearance of infectious HSV-1 in culture supernatants). While infectious virus was detected in none of the latently infected / non-stressed control cultures, reactivation occurred in 62.5 ± 3.5 % of TG cell cultures (n=72) following heat stress. No correlation was observed following heat stress between HSV-1 reactivation and either IL-6 or TNF- α levels in culture supernatants.

Effects of latent HSV-1 and heat stress on IL-6 production from TG cells. IL-6 expression was compared in latently infected and uninfected TG cell cultures following heat stress. Supernatants taken from latently infected cultures contained significantly less IL-6 than uninfected cultures at 0 (i.e. 24 hours after medium replacement) and 24 hours after heat stress (Table 3). Likewise in non-stressed cultures at these time points, IL-6 concentrations were lower in supernatants from latently infected TG cell cultures than uninfected cultures. By 48 hours after heat stress, IL-6 levels were equivalent in all four treatment groups (i.e. UI /NS, I /NS, UI /S, I/S). By 72 hours after heat stress, however, IL-6 levels from heat-stressed TG cell cultures was significantly lower than that of their non-stressed controls. (Table 3).

Dexamethasone suppresses IL-6 production from TG cells. Epinephrine and glucocorticoids (e.g., dexamethasone) induce reactivation of latent herpesviruses in animal models (Kwon et al., 1981; Rock et al., 1992). While epinephrine does not induce HSV-1 reactivation from latently infected TG cells, dexamethasone does so in a dose-dependent manner (Halford et al., 1996a). Because glucocorticoids suppress IL-6 production by leukocytes and astrocytes (Benveniste et al., 1990; Waage et al., 1990), the effects of dexamethasone on IL-6 production in TG cell cultures was assessed. Culture medium was replaced with medium containing either dexamethasone, epinephrine, or no drugs (i.e. vehicle),

and culture supernatants were collected every 24 hours thereafter over a 96 hour period. Dexamethasone (10^{-7} - 10^{-11} M) significantly suppressed IL-6 production at all time points, and did so in a dose-dependent manner (Figure 2). Ninety-six hours after replacing the culture medium, IL-6 concentrations in 10^{-7} M dexamethasone-treated cultures were approximately 20-fold lower than in untreated cultures. Epinephrine also suppressed IL-6 production, but only at the highest dose, and only at 72 and 96 hours after replacing culture medium (Figure 2).

Mechanism of dexamethasone-mediated IL-6 suppression in TG cell cultures.

Glucocorticoid hormones regulate gene expression both at the transcriptional and post-transcriptional levels (Waage et al., 1990; Ray et al., 1990; Schobitz et al., 1993; Zitnik et al., 1994). To determine if dexamethasone suppressed IL-6 production from TG cells by downregulating IL-6 mRNA transcription, reverse transcription PCR analysis was performed on 10^{-7} M dexamethasone versus vehicle-treated TG cell cultures. Fifteen hours after culture medium replacement, RNA was harvested from one pair (dexamethasone versus vehicle-treated) of latently infected TG cell cultures prior to heat stress. The remaining cultures were heat stressed, and RNA was harvested from the remaining culture pairs at 12, 24, and 48 hours after heat stress. Regardless of dexamethasone treatment, TNF- α mRNA expression was downregulated in TG cells by 24 and 48 hours after heat stress (Figure 3). There was no evident differences in IL-6 mRNA levels between dexamethasone and vehicle-treated cultures at 0, 12, 24, or 48 hours following heat stress (Figure 3). In contrast, 10^{-7} M dexamethasone induced HSV-1 reactivation in TG cell cultures prior to heat stress (i.e. t=0 hour) as demonstrated by the upregulation of HSV-1 infected cell polypeptide 27 (ICP27) mRNA transcription (Halford et al., 1996a). Moreover, increased levels of lytic phase mRNA transcripts (e.g. ICP27) were detected in dexamethasone-treated cultures 12 hours after heat stress, suggesting that activation of the glucocorticoid receptor facilitates heat stress-induced HSV-1 reactivation.

Corticosterone/dexamethasone-mediated suppression of IL-6 mRNA transcription is most evident 2-8 hours after treatment (Schobitz et al., 1993; Zitnik et al., 1994). To ensure that dexamethasone had not acted on IL-6 mRNA transcription prior to the collection of the first sample (i.e. 15 hours post-DEX), RT-PCR was performed on RNA from TG cells 2 hours after dexamethasone or vehicle treatment. No differences in IL-6 mRNA levels were evident by conventional RT-PCR (Fig. 4a), nor by competitive RT-PCR (Fig. 4b). Therefore, dexamethasone-mediated suppression of IL-6 production appears to occur at the post-transcriptional level in TG cells.

Previous studies suggest that both TNF- α and IFN- γ play a role in controlling reactivation of latent HSV-1 (Cantin et al., 1995; Liu et al., 1996). Although there was no indication that either cytokine was present in the TG cultures by ELISA, RT-PCR assays were performed to detect TNF- α and IFN- γ transcripts. However, neither cytokine transcript was detected in these cultures suggesting the absence of even minute amounts that would not be detected by the less-sensitive ELISA (data not shown).

Discussion

In the present study, cytokine levels were determined in TG cells obtained from HSV-1 latently infected and uninfected mice and cultured over a 20 day period. Of the T_H1 (IL-2 and IFN- γ), T_H2 (IL-10), and proinflammatory (IL-1 β , IL-6, and TNF- α) cytokines screened in the culture supernates, only IL-6 was consistently produced in significant amounts. The observation that hyperthermic exposure of cultures significantly reduced IL-6 production 72 hour post exposure is most probably due to the loss of cells following exposure at 43°C (unpublished observation) rather than an effect of heat shock proteins on IL-6 synthesis (Ensor et al., 1994). This notion is also supported by the data showing that hyperthermic stress had no effect on IL-6 mRNA levels over a 48 hour period. The decrease in TNF- α mRNA following hyperthermic stress is consistent with reports showing heat shock proteins suppress TNF- α production (Ensor et al., 1994; Hall, 1994). In those cultures that were not exposed to hyperthermic stress, the reduction in TNF- α could be due to the inhibitory effects of IL-6 on TNF- α production (Aderka et al., 1989; Schindler et al., 1990).

The considerable amount of IL-6 produced by the TG cells in culture may arise from the CD11b⁺ population which includes macrophages and microglial cells that are known to synthesize IL-6. Neurons that are present in these TG cell cultures may also be a source of IL-6 as recently reported using embryonic neuron cultures (Ringheim et al., 1995). However, production of IL-6 by the TG cell cultures is constitutive which is not consistent with convention in that IL-6 is typically elicited by proinflammatory cytokines (e.g., IL-1 β or TNF- α) or other stimuli (e.g., lipopolysaccharide). The mechanism of IL-6 production by the cultured TG cells is

currently unknown. However, we propose two explanations. Explants of whole TG removed from HSV-1 latently infected mice do not produce IL-6 in the absence of nerve growth factor measured over a 5 day period even though they possess IL-6 transcripts (Halford & Carr, unpublished observation). Whether nerve growth factor directly stimulates the cultured cells to secrete IL-6 is not known, although it has been reported that IL-6 stimulates the production of nerve growth factor by cultured astrocytes (Frei et al., 1989). Alternatively, the removal of TGs from the animals may allow the TG cells cultured over time to secrete IL-6 that *in situ* would be suppressed by corticosterone (Goujon et al., 1995).

Dexamethasone suppression of IL-6 production by TG cell cultures is consistent with the IL-6 gene possessing sequences that bind the glucocorticoid receptor complex (Ray et al., 1990). Moreover, the kinetics in the suppression of the IL-6 mRNA by dexamethasone parallels that previously reported measuring splenic and pituitary gland IL-6 transcripts (Schobitz et al., 1993). However, the data showing a qualitatively equivalent amount of IL-6 mRNA at 12-48 hour between dexamethasone (10^{-7} M) treated and untreated cultures yet a significant reduction in IL-6 protein levels in the dexamethasone-treated cultures suggests message instability as previously reported (Zitnik et al., 1994). Although dexamethasone (10^{-7} M) has been found to reactivate 20-25% of the TG cell cultures latently infected with HSV-1 (Halford et al., 1996a), there is no correlation with the cultures that reactivate and the levels of IL-6 produced. Consequently, the reactivation status of HSV-1 in TG cells *in vitro* is not directly linked to IL-6 production. The consideration of IL-6 protein levels is important since there is evidence suggesting that HSV-1 upregulates IL-6 gene expression in a permissive cell line (Kanangat et al., 1996). The data showing that the IL-6 levels are suppressed in the HSV-1 latently infected TG cells compared to the uninfected cells may be due to the presence of latency associated transcripts (Stevens et al., 1987) that non-specifically suppress cellular genes or the synthesis of the virion host shutoff protein (Zelus et al., 1996).

The presence of CD8⁺ cells in the majority of the HSV-1 infected TG cultures screened is supported by reports showing the presence of infiltrating CD8⁺ lymphocytes in TGs of HSV-1-infected mice monitored 1-6 months p.i. (Cantin et al., 1995; Liu et al., 1996; Shimeld et al., 1995). Curiously, the TG cell cultures did not contain CD4⁺ lymphocytes nor was there a significant difference in CD11b⁺ cells (defining macrophages, microglia, neutrophils, and NK cells) comparing HSV-1-latently infected to uninfected TG cultures. The absence of CD4⁺ cells is also reflected in the absence of lymphokines (e.g., IFN- γ , IL-2, and IL-10) that are detected in TGs from latently infected mice (Cantin et al., 1995; Liu et al., 1996; Halford et al., 1996b). The low to undetectable levels of TNF- α and IL-1 β in the TG cultures along with the lack of spontaneous reactivation monitored over 20 days would seem to suggest that the presence of these cytokines is not responsible for holding the virus in a latent state.

In conclusion, there does not appear to be a direct relationship between HSV-1 infection in TG cell cultures and IL-6 production. Dexamethasone-induced reactivation of latently infected TG cells *in vitro* did not correlate with the level of IL-6. Specifically, although the rate of hyperthermically-induced HSV-1 reactivation was faster in the dexamethasone-treated cultures, only 2/9 cultures reactivated with dexamethasone exposure only. However, a reduced expression of the 2.0 kb LAT transcript was observed in cultures treated with dexamethasone (Halford & Carr, unpublished observation). Potentially, dexamethasone down regulates LAT expression in neurons by blocking constitutive synthesis (Kaltschmidt et al., 1994) and translocation of NF κ B to the nucleus via I κ B induction (Auphan et al., 1995; Scheinman et al., 1995). Since NF κ B binding motifs are present in the 1 kb region upstream of the TATA box where LAT is initiated (Dobson et al., 1995), dexamethasone inhibition of NF κ B activity could potentially block LAT transcription. Currently, the function of LAT is unknown. However, there is evidence to suggest that LATs serve as antisense inhibitors of ICPO mRNA which favors the establishment of latency (for review, Halford and Carr, 1996. Future work using this *in vitro* model will address this hypothesis.

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Figure legends

Figure 1. TG cell cultures from HSV-1-infected mice contain CD11b⁺ and CD8⁺ cells.

TG cells obtained from HSV-1 latently infected and uninfected mice were cultured as described in the Materials and methods section. Cells were harvested on day 14 and examined for the presence of CD11b-, CD4- and CD8-positive cells by flow cytometry. This figure is representative of the findings summarized in Table 2 for CD8⁺ cells. Panel A, isotypic control; panel B, TG cells from uninfected mouse; panel C, TG cells from HSV-1 latently infected mouse.

Figure 2. Dexamethasone suppresses IL-6 production by TG cells. Supernates were collected from epinephrine (EPI)- and dexamethasone (DEX)-treated TG cell cultures just prior to (A), and 24 hr (B), 48 hr (C), and 72 hr (D) following hyperthermic stress. Drugs were added to TG cells 24 hours prior to heat stress. This figure is a summary of 2-4 experiments per treatment. * $p < .05$ comparing drug- to vehicle-treated group. Bars represent SEM, $n = 10-20$ /group.

Figure 3. Effects of dexamethasone on cytokine and HSV-1 mRNA transcription in TG cells. RT-PCR analysis of cytokine and viral transcripts was performed on RNA obtained from hyperthermally-stressed TG cell cultures at 0, 12, 24, and 48 hours after heat stress. Fifteen hours prior to heat stress, culture medium was replaced with medium containing either 10^{-7} M dexamethasone (DEX) or no drugs (VEH). RT-PCR was used to detect mRNAs for glyceraldehyde-3-phosphodehydrogenase (G3PDH), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α).

Figure 4. Competitive RT-PCR analysis of IL-6 mRNA. RNA was extracted from TG cells two hours after culture medium was replaced with medium containing either 10^{-7} M

dexamethasone (DEX) or no drugs (VEH). (A) RT-PCR analysis of TG cell RNA for G3PDH and IL-6. (B) Competitive PCR for G3PDH and IL-6 was performed on cDNA as described in Materials and Methods.

Table 1. Oligonucleotide RT-PCR primers.

PCR PRIMERS	SEQUENCE	PCR PRODUCT SIZE (bp)
G3PDH →	5'-gaa tct act ggc gtc ttc acc -3'	239
← G3PDH	5'-gtc atg agc cct tcc acg atg c-3'	
IL-6 →	5'-ttc cat cca gtt gcc ttc ttg g-3'	360
← IL-6	5'-ctt cat gta ctc cag gta g-3'	
TNF- α →	5'-gcc tgt agc cca cgt cgt ag-3'	384
← TNF- α	5'-ttg ggc aga ttg acc tca gc-3'	
ICP27 →	5'-ttt ctc cag tgc tac ctg aag g-3'	283
←ICP27	5'-tca act cgc aga cac gac tcg-3'	

Sense / antisense polarity of primer is indicated by '→' / '←', respectively.

Table 2. TG cell cultures possess CD11b⁺ and CD8⁺ cells^a.

TG CELL CULTURES	CD4⁺	CD8⁺	CD11b⁺
Uninfected	0	0.3 ± 0.2 ^b	8.0 ± 2.0
Infected	0	0.92 ± 0.4	5.2 ± 1.4

^aCells from TG cultures were phenotypically characterized for the surface expression of CD4, CD8, and CD11b antigen.

^bNumbers are in mean percent positive ± SEM, n=5/group.

Table 3. HSV-1 infected TG cells produce less IL-6 protein^a.

GROUPS	0 h	24 h	48 h	72 h
Uninfected, (UI / NS) not stressed	20.5 ± 2.3 ^b	80.6 ± 13.7	113 ± 11.6	241 ± 30.2
Infected, (I / NS) not stressed	12.1 ± 1.9 [*]	45.4 ± 8.6 [*]	124 ± 20.9	275 ± 16.0
Uninfected, (UI / S) heat stressed	22.9 ± 2.9	56.9 ± 12.0	96.3 ± 20.1	132 ± 16.3 [*]
Infected, (I / S) heat stressed	12.2 ± 1.6 [*]	26.5 ± 1.9 [*]	108 ± 13.2	177 ± 25.7 [*]

^aSupernates were collected 0, 24, 48, and 72 hr after heat stress of TG cell cultures. IL-6 was detected by ELISA. This table summarizes the results of two independent experiments.

^bNumbers are in ng/ml; mean ± SEM, n=10/group.

^{*}p<.05 comparing infected to uninfected cultures treated under the same conditions except the 72 hr time point in which heat-stressed versus non-stressed controls were compared as determined by ANOVA and Tukey's post hoc t-test.

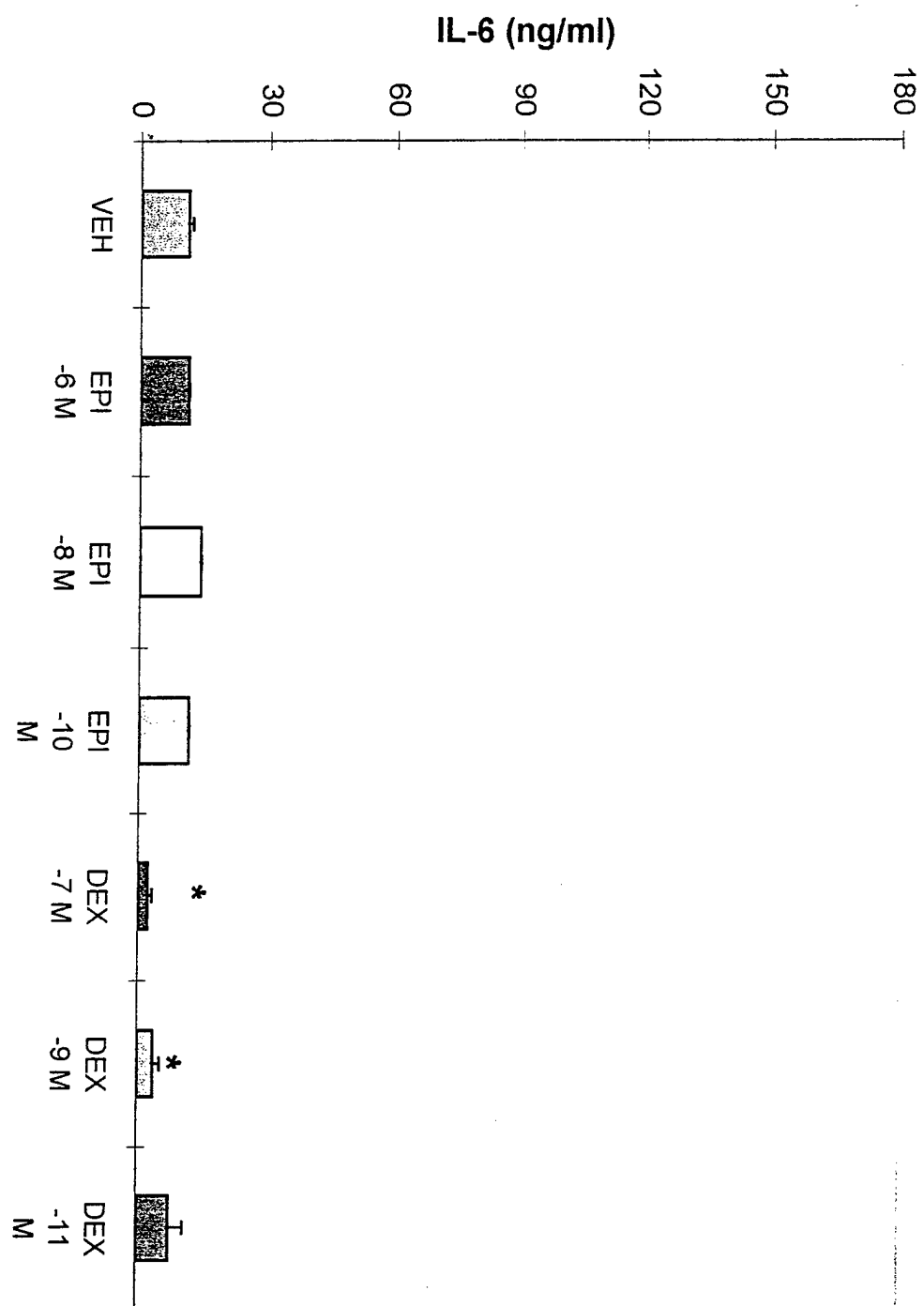


FIGURE 2A

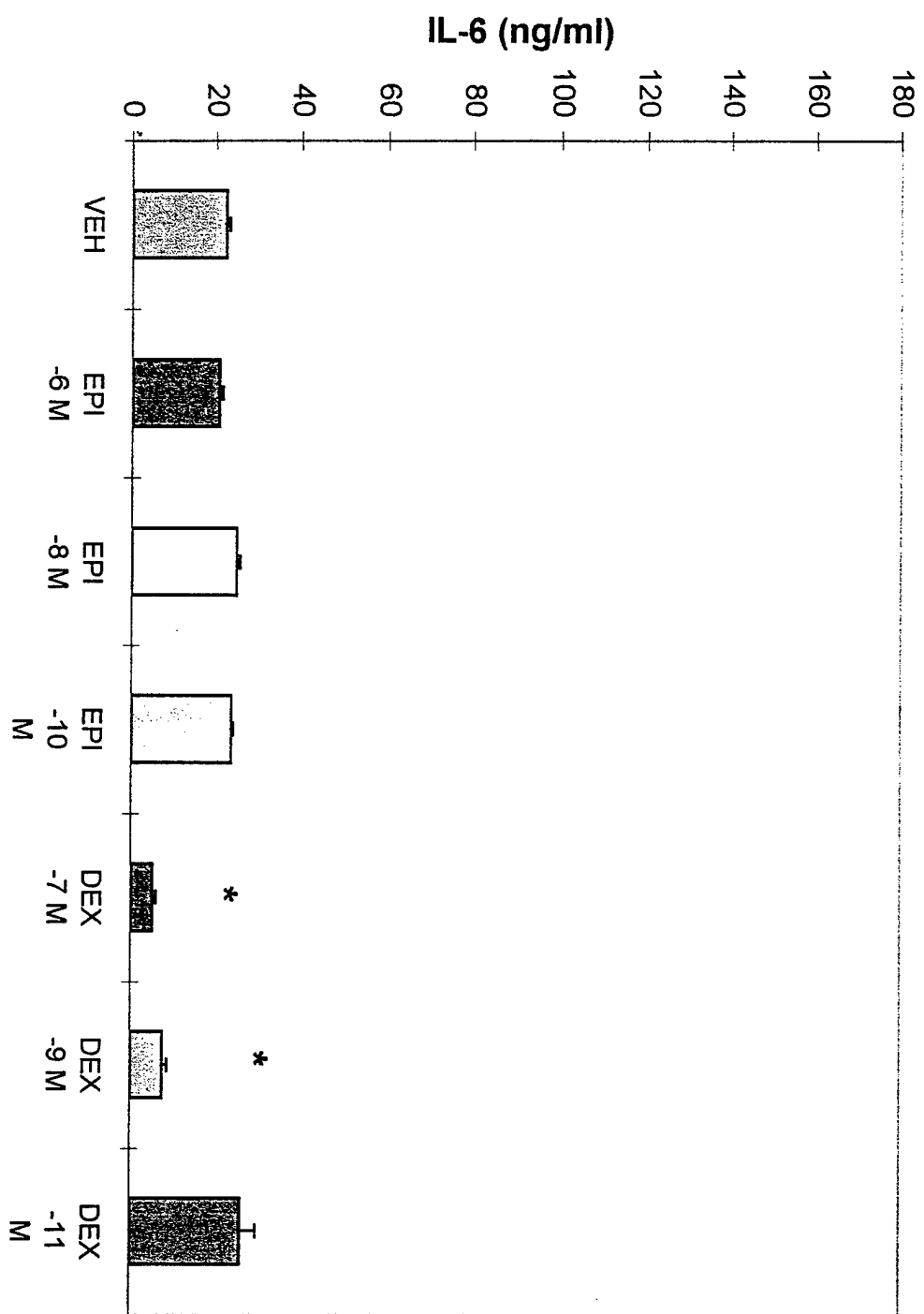


FIGURE 2B

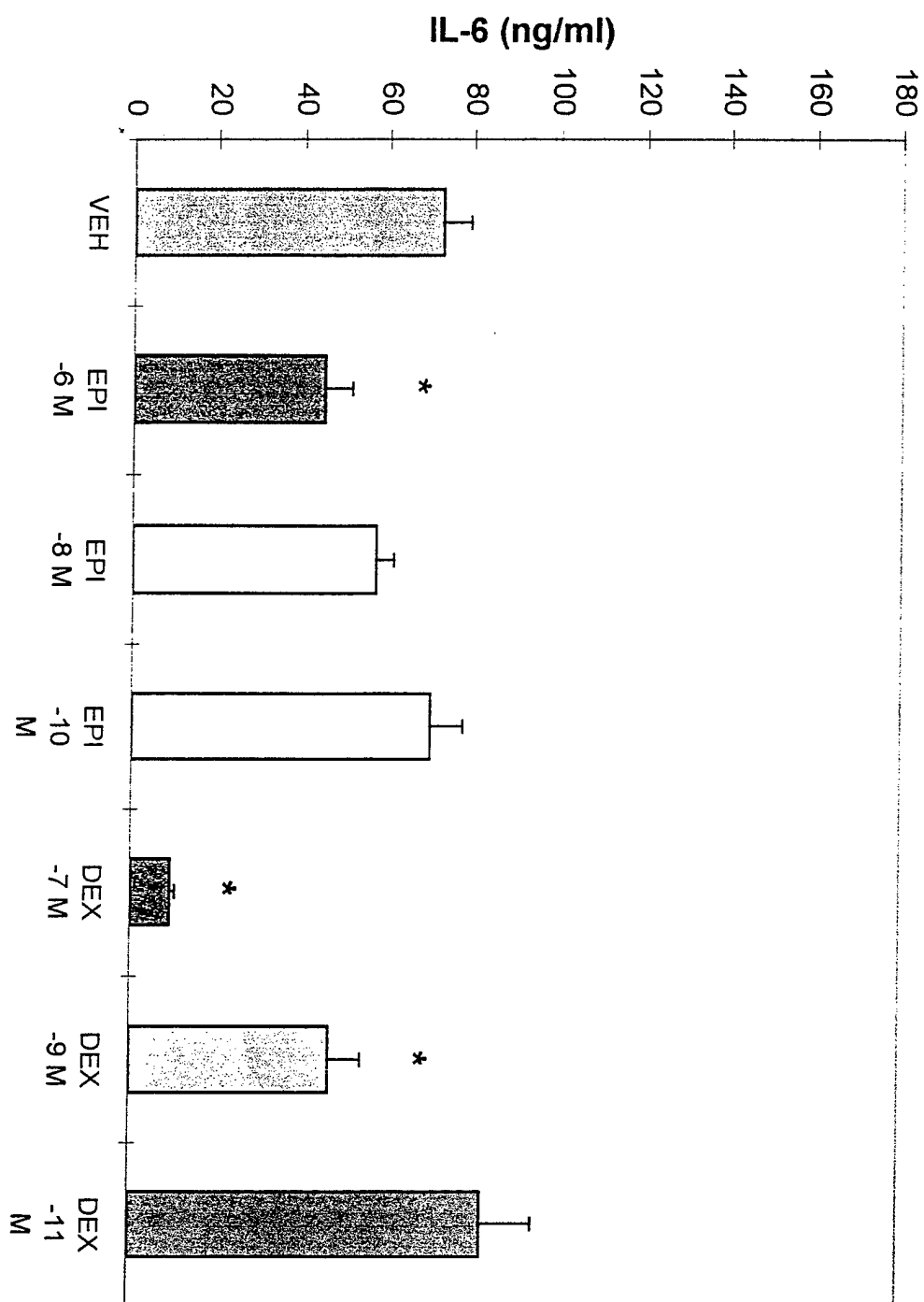


FIGURE 2C

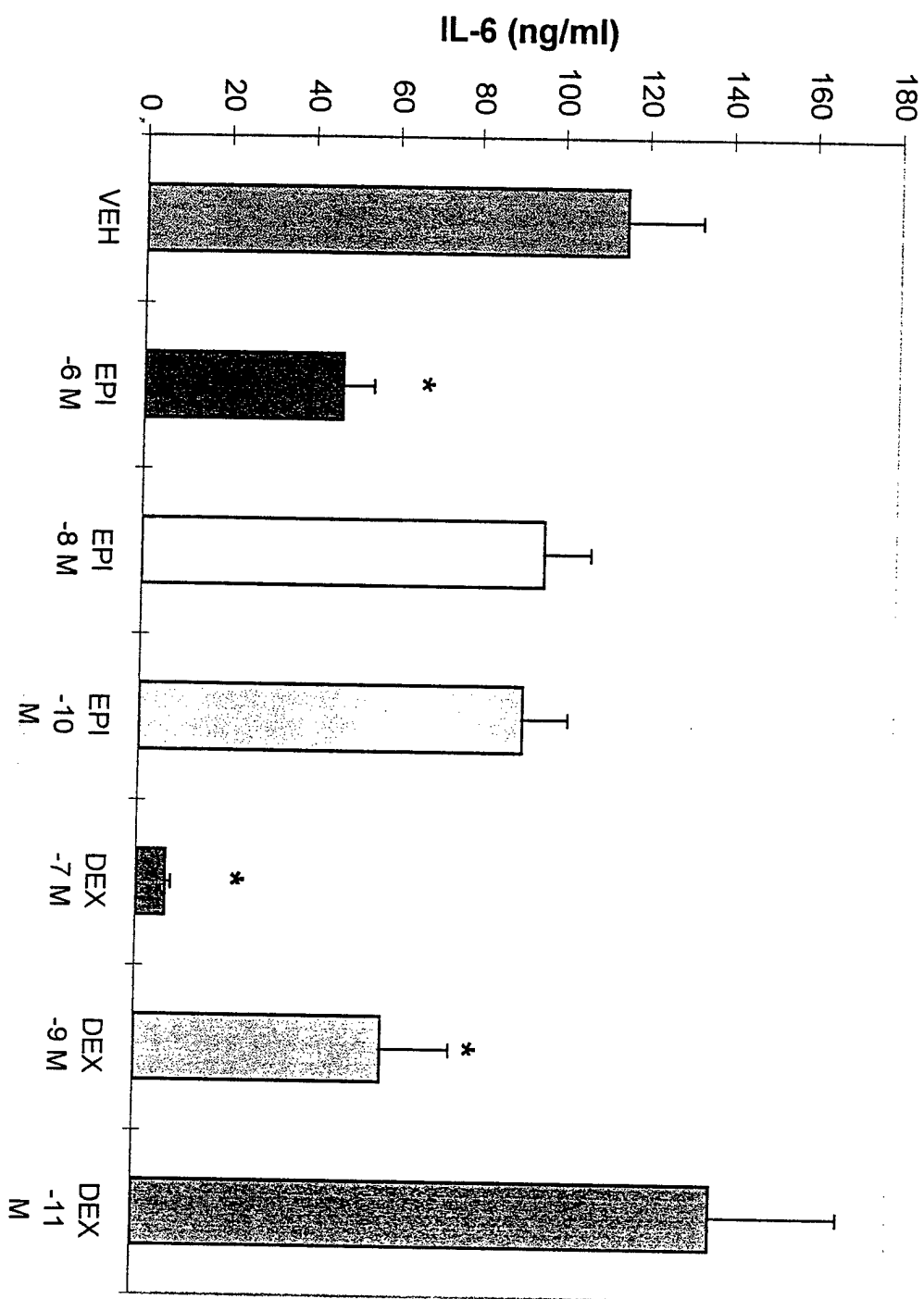


FIGURE 2D

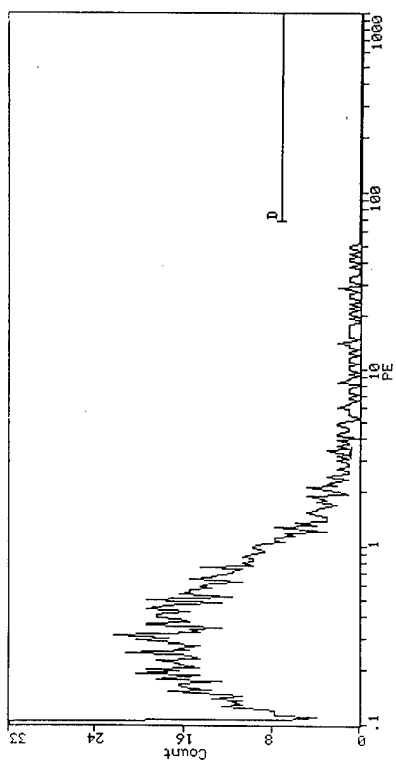


FIGURE 1A

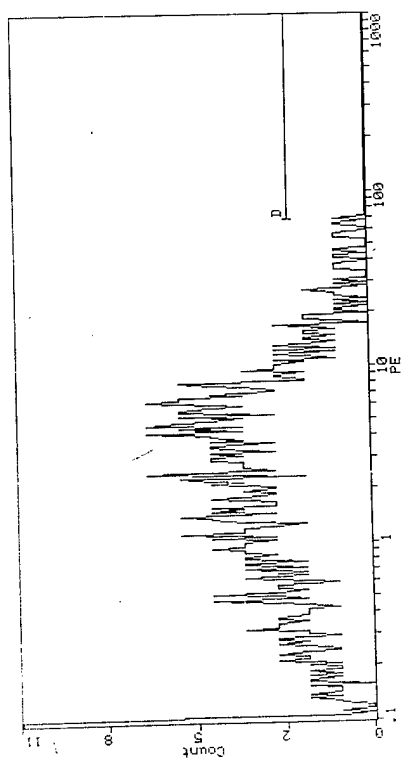


FIGURE 1B

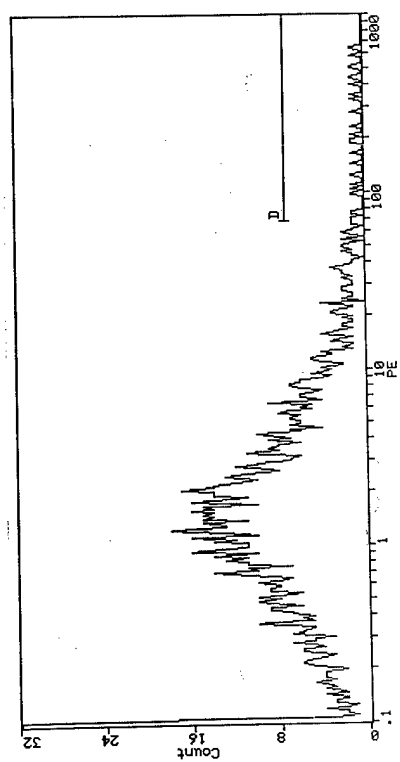


FIGURE 1C

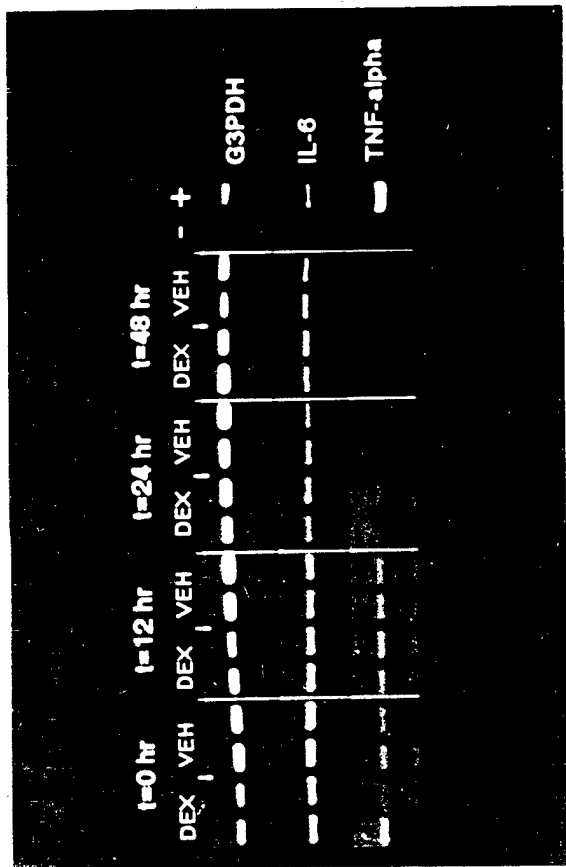


FIGURE 3

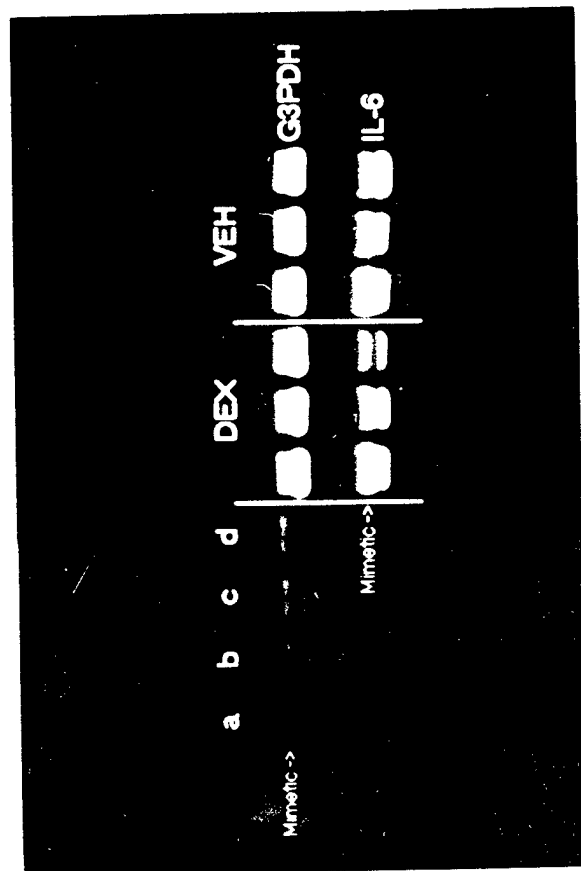


FIGURE 4B

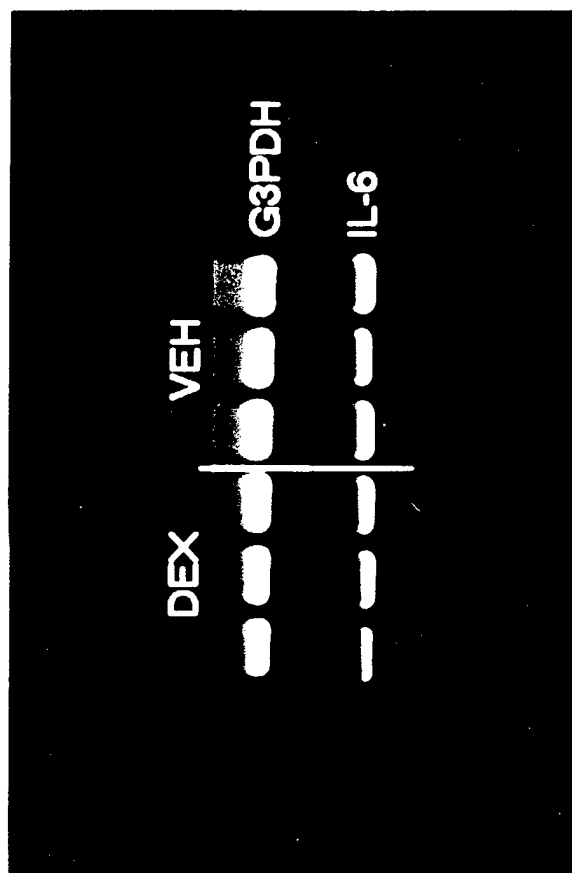


FIGURE 4A

**Corticosterone is not Associated with Acute Morphine-Mediated Suppression of
Peritoneal Cytotoxic T Lymphocyte Activity in Alloimmunized C3H/HeN Mice¹**

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ABSTRACT

Morphine is an immunomodulatory compound suppressing both humoral and cell-mediated immunity following *in vivo* administration. Both the hypothalamic pituitary adrenal (HPA) axis and adrenergic processes have been implicated in morphine-mediated immunomodulation. To further study the HPA axis relative to morphine-induced immunosuppression, corticosterone levels were measured at various times following morphine (50.0 mg/kg) administration. Whereas corticosterone levels were elevated in morphine-treated mice 4 hrs post drug administration, there were no differences in the levels compared to vehicle-treated mice 120 hrs post drug administration and alloimmunization. However, acute morphine (50 mg/kg, s.c.) administration suppressed (50%) peritoneal Cytotoxic T lymphocyte (CTL) activity in alloimmunized mice suggesting that the initial rise in corticosterone might be responsible for the suppression in CTL activity following alloimmunization. However, cyanoketone (10 mg/kg) administered to mice daily for 4 consecutive days significantly elevated (3 fold) serum corticosterone levels immediately prior to alloantigen immunization yet, there was no effect on splenic or peritoneal CTL. In addition, mice receiving the cyanoketone treatment showed an elevation in corticosterone 120 hr post alloimmunization and such an increase was blocked in the presence of morphine (50 mg/kg). However, when mice previously treated with cyanoketone (10 mg/kg/day for 4 days) were administered morphine (50 mg/kg, s.c.), there was no change in the suppressive effect (50%) of morphine on peritoneal CTL activity. Collectively, the results suggest that morphine-mediated suppression of peritoneal CTL activity in alloimmunized C3H/HeN mice is not mediated through the HPA axis and corticosterone.

key words: morphine, cyanoketone, corticosterone, cytotoxic T lymphocyte

INTRODUCTION

Acute and chronic administration of morphine has been shown to suppress natural killer (NK) activity (Shavit et al., 1984), mitogen-induced lymphocyte proliferation (Bryant et al., 1987), antibody production (Bussiera et al., 1993), phagocytosis (Szabo et al., 1993), and allogeneic immune responses (Carpenter et al., 1994; Maity et al., 1995). By definition, these effects are mediated by opioid receptors in that opioid antagonists block the action of morphine. Although morphine (i.e., μ)-type binding sites have been identified on cells of the immune system using binding studies (Makman et al., 1995), affinity-labeling techniques (Radulescu et al., 1991), and molecular cloning techniques (Chuang et al., 1995), the immunosuppressive action of morphine has been shown in part to be mediated by central (brain) pathways (Shavit et al., 1986) involving the HPA axis (Bryant & Roudebush, 1990; Bryant et al., 1991; Sei et al., 1991). Recently, the glucocorticoid receptor antagonist RU 38486 has been shown to block morphine-mediated suppression of NK activity (Freier & Fuchs, 1994). To assess the potential involvement of the HPA axis on morphine-mediated suppression of peritoneal CTL activity, cyanoketone was employed using alloimmunized mouse previously shown to exhibit suppressed peritoneal CTL activity following an acute administration with morphine (Carpenter et al., 1996).

An optimal concentration (60-100 mg/kg administered daily for 2-4 days) of cyanoketone blocks corticosterone synthesis by binding to the enzyme 3β -ol-dehydrogenase- Δ^5 , Δ^4 -isomerase and has been used to study glucocorticoid responses to stress (Akana et al., 1983; Akana et al., 1992) and HPA axis involvement in tumor necrosis factor- α production (Fantuzzi et al., 1995). However, the results of the present study suggest that the HPA axis does not have a significant role in the suppression of peritoneal CTL activity following an acute exposure of mice to morphine.

METHODS

Mice and tumor lines

Female C57BL/6 and C3H/HeN mice (Harlan-Sprague Dawley, Indianapolis, IN) 20-24 grams were housed in groups of 2-6 per cage and maintained on a 12 hr light/dark cycle. Access to food and water was *ad libitum*. Animals used in these studies were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, Department of Health, Education, and Welfare Publication Number (NIH) 85-23, revised 1985.

The YAC-1 mouse lymphoma line and EL-4 lymphoma cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD); the EL-4 and YAC-1 cells have been maintained in culture by biweekly passage over a 5-6 month period.

Splenocyte and peritoneal exudate cell (PEC) preparation

All mice were sacrificed by CO₂ asphyxiation and peritoneal lavage was performed using 10 ml of Hank's balanced salt solution (HBSS). Cells were collected by recovery of peritoneal lavage fluid through a 20 gauge needle and a 10 ml syringe. Spleens were surgically removed and cell suspensions were prepared by mechanical dispersion. Splenocytes and PEC were washed with HBSS (250 x g, 5 min) and triturated in 1 ml of 0.84% NH₄CL for 2 min. Cells were subsequently washed with 7 ml of HBSS (250 x g, 5 min) and resuspended in RMPI-1640 medium (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (Gibco) and 2.5% Hybri-max (Sigma Chemical Co., St. Louis, MO) antibiotic/antimycotic solution (hence forth, complete medium). Cells were counted and examined for viability using trypan blue.

⁵¹Cr-release cytolytic assay

Splenocytes were assayed using a 4-hr ⁵¹Cr-release microcytotoxicity assay as previously described (Carpenter et al., 1994). YAC-1 cells were used as targets to measure NK activity in the splenic population while EL-4 (H-2^b haplotype) cells were used as targets to measure CTL activity in the splenic and PEC populations. Numbers are expressed in lytic units. One lytic unit is defined as the reciprocal to the number of cells required to lyse 20% of the targets expressed per 10⁷ total cells.

Serum corticosterone and adrenocorticotropin hormone (ACTH) assays

Animals were sacrificed at the indicated time point by CO₂ asphyxiation and the thoracic cavity immediately opened and blood extracted via heart puncture with a 1 ml syringe and 27 1/2 gauge needle. Samples were placed on ice overnight and exposed to the air to favor clot formation. Tubes were then centrifuged (10,000 x g, 1 min) and sera removed and stored at -20°C for later analysis. All samples were collected between the hours of 8-10 AM. Sera from sacrificed animals were assayed for corticosterone and ACTH by radioimmunoassay (ICN Biomedicals, Costa Mesa, CA). All samples were assayed in duplicate. Correlation coefficients for the standard curves typically exceeded -.9900.

Experimental design

In the first experiment, C3H/HeN female mice (n=6/group) received 10.0 mg/kg cyanoketone or vehicle (dimethyl sulfoxide, DMSO) s.c. daily for 4 days. This dose had been found to be effective in blocking lipopolysaccharide-induced elevations in serum corticosterone (Fantuzzi et al., 1995). On the fifth day, the mice were immunized with 1 X 10⁷ C57BL/6 splenocytes i.p. Five days following alloimmunization, the mice were sacrificed and splenic NK and PEC and splenic CTL activity were determined. In addition, sera was obtained and assessed for corticosterone and ACTH levels.

In the second experiment, C3H/HeN female mice (n=5/group) were administered with 10.0 mg/kg cyanoketone or vehicle s.c. daily for 4 days. On the fifth day, the mice received 50.0 mg/kg of morphine or vehicle (HBSS) s.c. 2 hr prior to receiving 1×10^7 C57BL/6 splenocytes i.p. Five days following the alloimmunization, the mice were sacrificed and peritoneal lymphocytes were assayed for CTL activity. In addition, sera was collected and assayed for corticosterone levels.

In a separate experiment, C3H/HeN female mice (n=8/group/timepoint) were administered 50.0 mg/kg of morphine or vehicle s.c. 2 hr prior to alloimmunization with 1×10^7 C57BL/6 splenocytes i.p. Two hr, 12 hr, and 120 hr post-alloimmunization, groups of mice were sacrificed and sera collected and assayed for corticosterone.

In the fourth experiment, C3H/HeN mice (n=6/group) were treated with cyanoketone (10.0 mg/kg) or vehicle i.p. daily for 4 days. On the fifth day, the mice were sacrificed and sera assayed for corticosterone levels.

Reagents

Morphine sulfate was provided by the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). Cyanoketone was provided by Sterling Winthrop (Collegeville, PA).

Statistics

One-way analysis of variance was used together with Tukey's t-test to determine significance ($p < .05$) between vehicle- and drug-treated groups. The statistical package used the GBSTAT program (Dynamic Microsystems Inc., Silver Springs, MD).

RESULTS

Cyanoketone has no effect on splenic NK, splenic CTL or peritoneal CTL activity.

Glucocorticoids are potent immunomodulatory compounds suppressing a variety of immune parameters including interleukin (IL)-2 and interferon (IFN)- γ production (Munck & Guyre, 1991) most probably through the induction of κ B synthesis (Scheinman et al., 1995; Auphan et al., 1995). Since the HPA axis and consequently glucocorticoid production would be activated during an immune response (Wick et al., 1993), it was predicted that suboptimal doses of cyanoketone would modify CTL activity in alloimmunized mice. However, mice exposed to cyanoketone (10.0 mg/kg, i.p.) daily for 4 consecutive days prior to alloimmunization showed no effect in the generation of CTLs or NK activity (Table 1). However, they did exhibit an elevation in corticosterone but not ACTH serum levels at the time of sacrifice (Table 2). Perhaps more intriguing, animals receiving the suboptimal cyanoketone-treatment regimen had higher serum levels of corticosterone compared to vehicle-treated controls (Fig. 1) immediately prior to alloimmunization.

Cyanoketone does not block morphine-mediated suppression of peritoneal CTL activity.

Previously, it has been shown that the acute administration of morphine (50.0 mg/kg, s.c.) suppresses peritoneal but not splenic CTL activity in a naltrexone-reversible manner (Carpenter et al., 1996). Morphine-mediated suppression of CTL activity might be due to the activation of the HPA axis resulting in increases in corticosterone similar to what was reported following acute morphine suppression of NK activity (Freier & Fuchs, 1994). To characterize the corticosterone response following a single exposure to morphine (50.0 mg/kg, s.c.) in alloimmunized mice, alloimmunized animals were sacrificed at 4, 12, and 120 hr post morphine administration and serum corticosterone levels measured. The results show an initial rise (4 hr), then subsequent fall (12 hr) prior to a leveling off (120 hr) in serum corticosterone following morphine administration (Fig. 2). Based on these results, cyanoketone and morphine were

administered to mice together to determine a predicted synergistic effect of the drugs (based on the elevation in serum corticosterone). Mice pretreated with cyanoketone showed a similar level of suppression in peritoneal CTL activity following acute morphine treatment compared to those animals received morphine alone (Fig. 3). However, the elevation in corticosterone observed in the cyanoketone-treated mice 120 hr post alloimmunization (Table 2) was not observed in mice receiving a combination of morphine and cyanoketone (Fig. 4). In addition, there were no changes in circulating ACTH levels in the morphine- or morphine and cyanoketone-treated mice compared to vehicle-treated animals (Fig. 4).

DISCUSSION

In the present study, the hypothesis that the HPA axis might, in part, be responsible for the suppression in peritoneal CTL activity following acute morphine administration in alloimmunized mice was tested. Both morphine and small doses (10 mg/kg) of cyanoketone were found to elevate corticosterone during the alloantigen immunization. Whereas morphine was found to be immunosuppressive, cyanoketone had no suppressive effect on CTL activity suggesting that the elevation in serum corticosterone levels is not linked to CTL suppression in the alloimmunized mice. These results are consistent with another recent finding in which it was found that only when mice were administered corticosterone (16 mg/kg/day) starting 4 days prior to alloantigen immunization (and not simultaneously with alloantigen immunization) was a significant suppression observed in CTL activity (DeKrey & Kerkvliet, 1995). Likewise, the involvement of the HPA axis in morphine-mediated suppression of mitogen-induced lymphocyte proliferation has recently been a focus of study. Specifically, the placement of morphine in the anterior hypothalamus resulting in the inhibition of concanavalin A-induced lymphocyte proliferation was not associated with an increase in plasma corticosterone levels (Hernandez et al., 1993). In addition, peripheral blood mononuclear cells from animals that were treated with RU486, adrenalectomized, or hypophysectomized prior to morphine

administration also showed inhibition of mitogen-elicited proliferation (Flores et al., 1994) suggesting that factors outside of the HPA axis are responsible for morphine-induced suppression of cell-mediated immune events.

Previous studies have shown a relationship between adrenergic pathways and morphine-mediated immunosuppression (Baddley et al., 1993; Carr et al., 1993; Fecho et al., 1993; Carr et al., 1994). Moreover, β -adrenoceptor stimulation has been shown to inhibit CTL activity (Strom et al., 1973). Since peripheral immune organs (i.e., lymph nodes and spleen) are innervated by sympathetic neuronal processes (Felten et al., 1985) that may act to regulate immunocompetence (Kruszewska et al., 1995), it is tempting to speculate that the activation of the sympathetic/adrenergic pathways following acute morphine administration is primarily responsible for the suppression in CTL activity in alloimmunized mice. However, the complexity of the brain-immune axis warrants further studies to fully elucidate the mechanism(s).

The observation showing 10 mg/kg of cyanoketone over a 4 day treatment regimen elevates serum corticosterone levels is surprising. A previous study found that 10 mg/kg of cyanoketone administered 24 and 2 hr prior to lipopolysaccharide treatment blocked (50%) the elevation in serum corticosterone (Fantuzzi et al., 1995) elicited by the endotoxin. Since the study by Fantuzzi and colleagues showed vehicle-treated corticosterone levels of 44 ng/ml using corn oil as the vehicle and the present study showed 150 ng/ml using DMSO as the vehicle, the present study suggests that the animals might have been under some form of stress as a result of the vehicle (DMSO) administration. This difference may, in part, explain the changes in corticosterone observed between this study and that conducted by Fantuzzi et al. (1995).

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Suboptimal treatment with cyanoketone increases serum corticosterone levels. C3H/HeN mice (n=6/group) were administered vehicle (DMSO) or 10.0 mg/kg cyanoketone i.p. daily for 4 days and sacrificed on day 5. Sera was obtained and assayed for corticosterone levels. * $p < .05$ comparing cyanoketone- to vehicle-treated group. Bars represent standard

error of the mean. This figure represents a summary of two independent experiments with n=3 mice/group/experiment.

Figure 2. Morphine increases serum corticosterone levels 4 hrs post administration.

C3H/HeN mice (n=8/group/time point) were administered vehicle (V, HBSS) or morphine (M, 50.0 mg/kg, s.c.) 2 hr prior to the administration of 1×10^7 C57BL/6 splenocytes i.p. At the designated time points, the mice were sacrificed and serum collected and assayed for corticosterone levels. *p<.05 comparing morphine- to vehicle-treated group. Bars represent standard error of the mean. This figure represents a summary of two independent experiments with n=4 animals/group/experiment.

Figure 3. Morphine suppresses PEC CTL activity.

C3H/HeN mice (n=5/group) were administered vehicle (DMSO) or cyanoketone (10.0 mg/kg) i.p. daily for 4 consecutive days. On the fifth day, the mice received morphine (50.0 mg/kg) or vehicle (HBSS) s.c. 2 hr prior to immunization with 1×10^7 C57BL/6 splenocytes. Five days following the alloimmunization, the mice were sacrificed and PEC CTL activity determined. *p<.05 comparing drug- to vehicle-treated mice. Bars represent standard error of the mean. This figure is a summary of two independent experiments using 2-3 mice/group/experiment.

Figure 4. Morphine blocks cyanoketone-induced augmentation in serum corticosterone.

C3H/HeN mice (n=5/group) were treated as described under the legend of figure 3. Upon sacrifice, serum was collected and assayed for corticosterone (a) and ACTH (b). Bars represent standard error of the mean.

Table 1. Cyanoketone Does not Modify Splenic NK or Splenic or PEC CTL Activity^a

Treatment	Splenic NK	Splenic CTL	PEC CTL
Vehicle	10.5 +/- 2.4 ^b	16.3 +/- 3.2	33.4 +/- 3.4
Cyanoketone	16.5 +/- 2.2	16.8 +/- 0.7	34.9 +/- 5.7

^aC3H/HeN (n=6/group) were administered vehicle (DMSO) or cyanoketone (10.0 mg/kg) i.p. daily for 4 consecutive days. On the fifth day, mice were alloimmunized with 1×10^7 C57BL/6 splenocytes. Five days following the alloimmunization, the mice were sacrificed and NK and CTL activity measured. This table is a summary of two independent experiments with n=3/group/experiment.

^bNumbers are displayed in mean lytic units₂₀ +/- standard error of the mean.

Table 2. Corticosterone Levels are Elevated in Cyanoketone-Treated Mice^a

Treatment	Serum Corticosterone (ng/ml)	Serum ACTH (pg/ml)
Vehicle	118 +/- 29 ^b	27.9 +/- 7.2
Cyanoketone	263 +/- 45*	20.0 +/- 1.7

^a C3H/HeN mice (n=6/group) were treated as described in table 1 legend. Five days following alloimmunization, the mice were sacrificed and serum corticosterone and ACTH levels were measured. This table is a summary of two independent experiments with n=3/group/experiment.

^bNumbers are in mean +/- standard error of the mean.

*p<.05 comparing the drug- to vehicle-treated group.

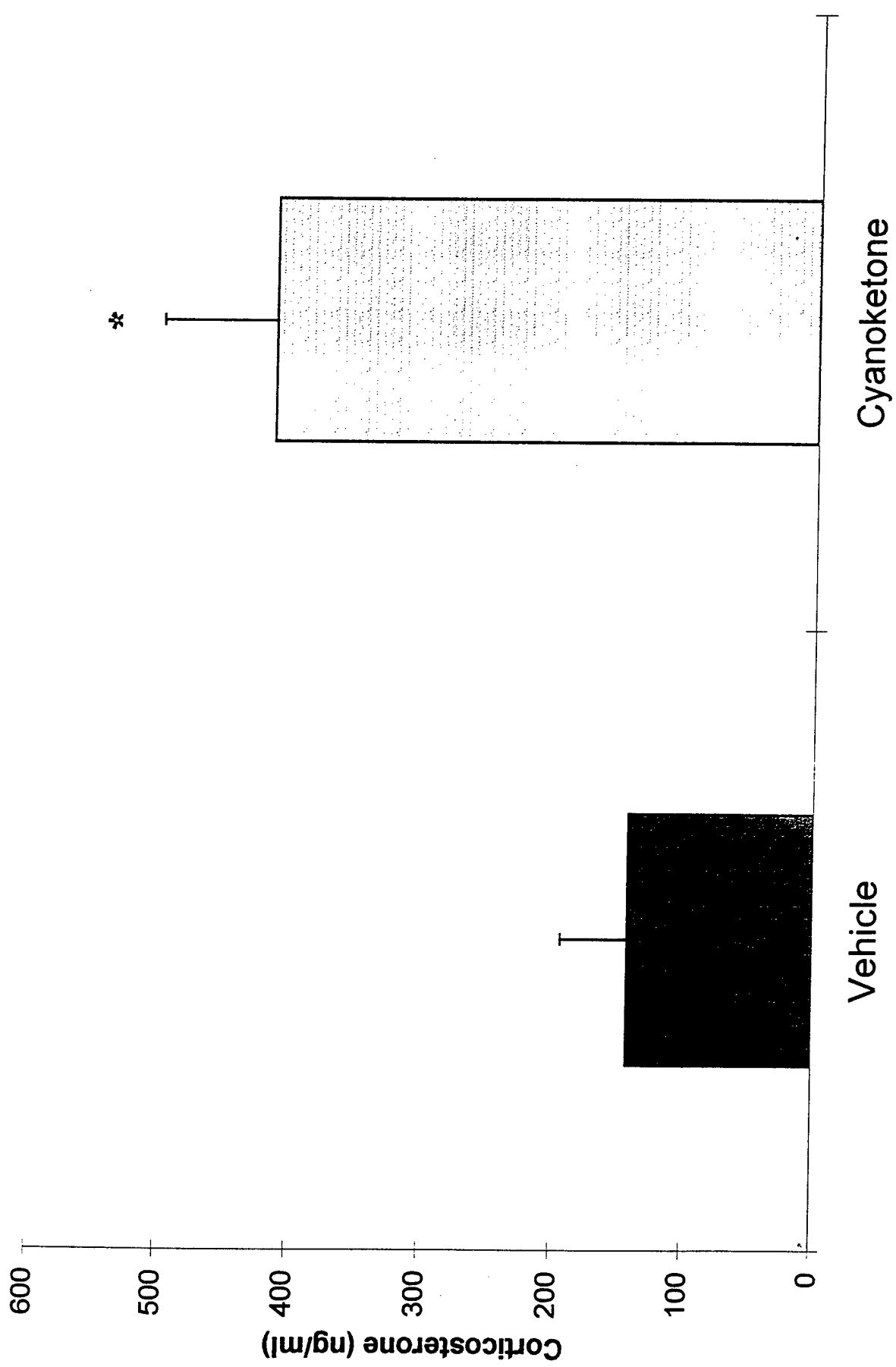
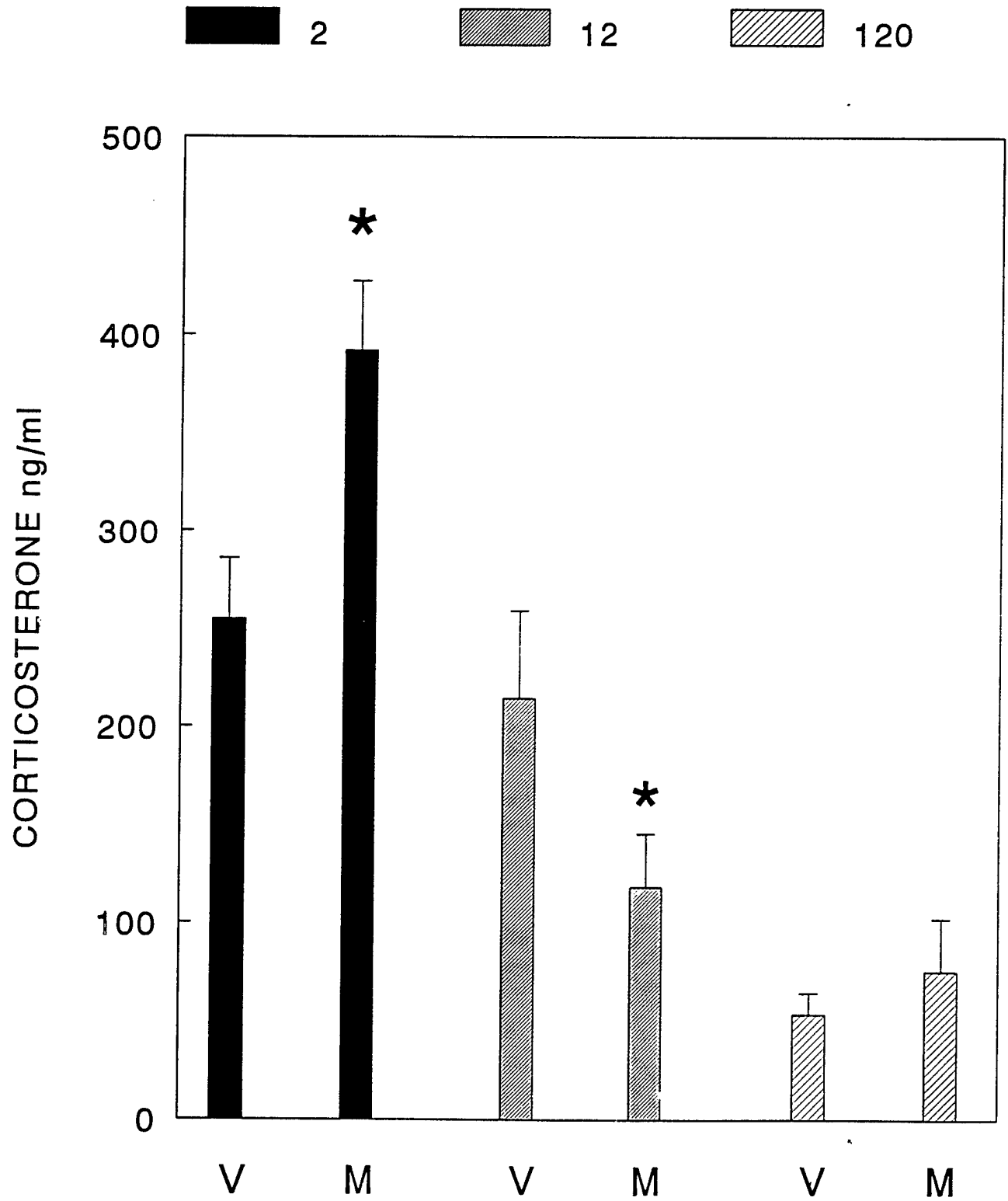


FIGURE 1

FIGURE 2



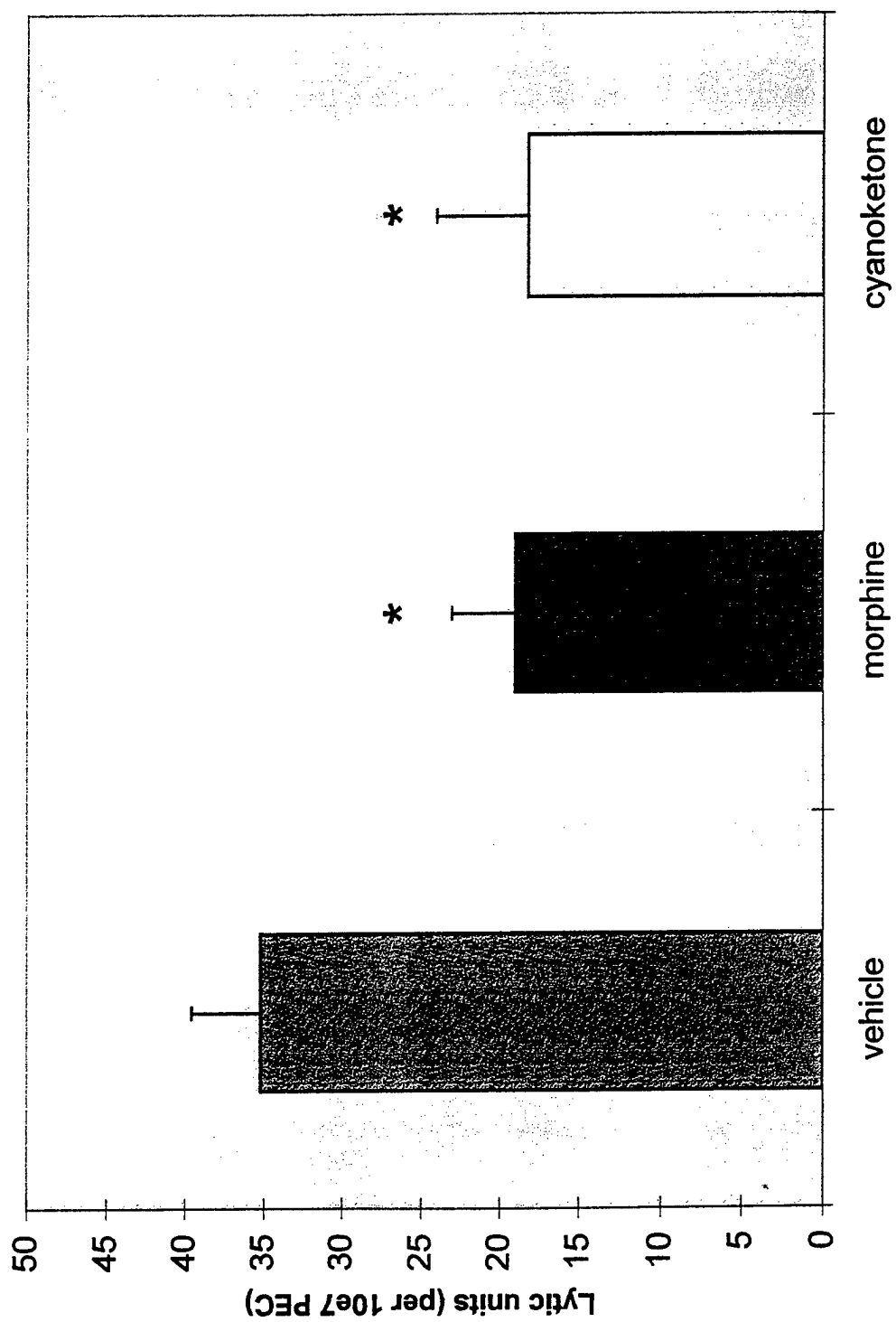


FIGURE 3

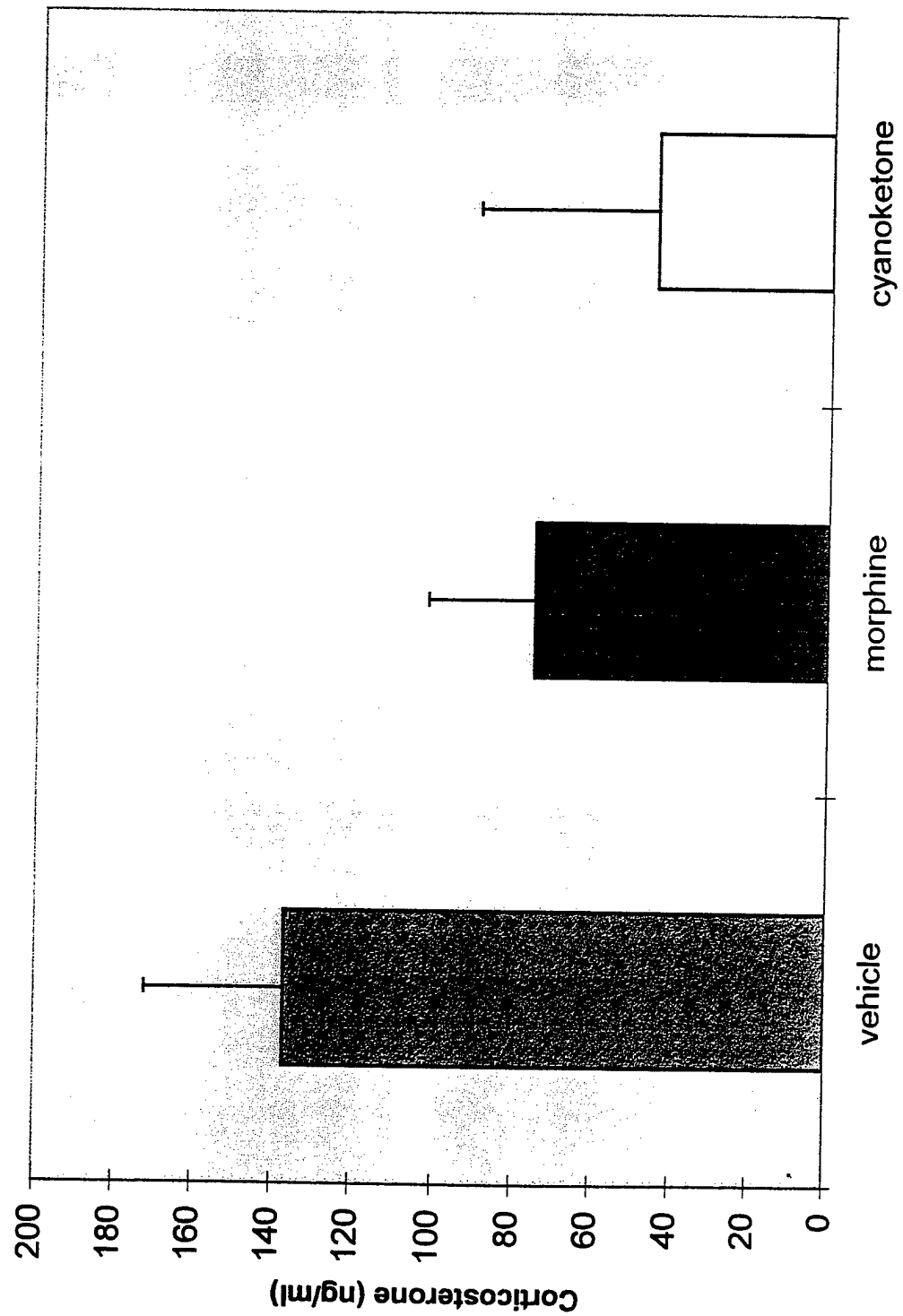


FIGURE 4

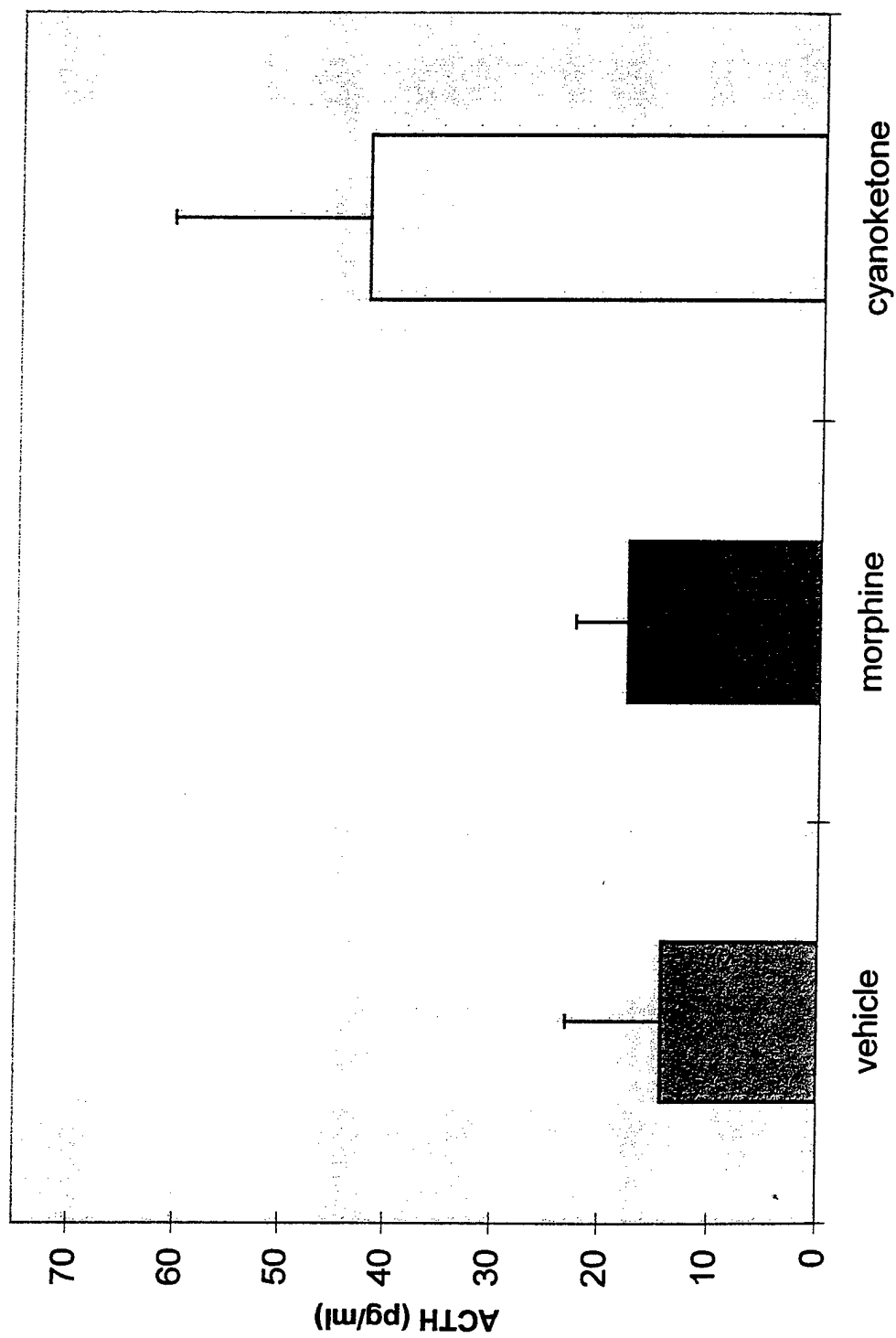


FIGURE 5

NEUROIMMUNOMODULATION

POSTER ABSTRACT FORM

LYMPHOCYTES DELAY KINETICS OF HSV-1 REACTIVATION FROM *IN VITRO* EXPLANTS OF LATENTLY INFECTED TRIGEMINAL GANGLIA (TG).

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Studies conducted in our lab with latent HSV-1-infected mice have shown the presence of cytokine transcripts in the trigeminal ganglia along with infiltrating mononuclear cells located at specific foci around neurons. To further these observations, an *in vitro* system was established in order to assess the potential of primed and naive lymphocytes to suppress reactivation of HSV-1 from TG explant cultures. In the presence of either naive or primed lymphocytes, the kinetics of reactivation from TG explant cultures proceeded more slowly as evidenced by a reduction in viral titer and the delayed appearance of CPE in the co-culture wells. To determine if cytokines were potentially involved in the anti-viral effect in cultures containing primed and naive lymphocytes, supernatants were collected and assayed for cytokine content by ELISA as well. The results suggest that cultures containing either naive or primed lymphocytes produce significant ($p < .05$) levels of IL-2 and IL-10. However, only those cultures that contained primed lymphocytes produced significantly ($p < .05$) elevated levels of IL-6 in samples taken 72-96 hrs post lymphocyte addition. There were no detectable levels of IFN- γ in any of the cultures. In the absence of TGs, there was no detectable IL-2, IL-6, IL-10 or IFN- γ in the supernatants of CV-1 monolayers in the presence of naive or primed lymphocytes indicating that the cytokines produced are due to the presence of the HSV-1-infected TGs. These results reflect the anti-viral effect of cytokines by direct or indirect means.

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